THE TERATOGENIC EFFECTS OF HYPERVITAMINOSIS A ON THE FORMATION OF THE NEURAL TUBE

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE AAN DE KATHOLIEKE
UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF. DR. P. G. A. B.
WIJDEVELD, VOLGENS HET BESLUIT VAN HET COLLEGE
VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN
OP MAANDAG 23 JUNI 1980 DES NAMIDDAGS TE
4 UUR

door

JOHANNES ANTONIUS GERARDUS GEELEN

geboren te Roermond

1980
krips repro meppel

The investigations for this thesis were performed at

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The Department of Anatomy, Medical School, University of Virginia, Charlottesville, Va , U S A

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Aan mijn ouders
Corry en Daphne

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INTRODUCTION

The formation of the neural tube is a crucial event during embryonic development of the nervous system. Disturbances of this process lead to serious congenital malformations such as anencephaly and spina bifida. These abnormalities which result from failure of the closure of the neural tube are among the most frequent congenital malformations of the central nervous system in humans.

The present thesis is a collection of six published articles which mainly concentrate on the normal and abnormal closure of the neural tube in embryos of mice and rats.

At first an article is presented which reviews the literature on congenital malformations induced by maternal treatment with excess vitamin A. The paper indicates that this treatment interferes with various developmental processes and that it is very effective in inhibiting the formation of the neural tube. Therefore it was decided to use this substance to elucidate some of the processes that are involved in the abnormal development of the nervous system.

In the next part of this thesis the normal closure of the neural tube is examined in the head region of the mouse embryo. The results of these light microscopic and ultrastructural studies are presented in two papers.

The next two articles present the results of our experiments with vitamin A. In the first one the effects of maternal administration of excess vitamin A on the cephalic mesenchyme is examined. The next one focuses on changes in the cephalic neuroepithelium of slightly older embryos with failure of neural tube closure.

Since failure of neural tube formation occurs very early in pregnancy we wished to find out how the abnormal nervous system continues to develop during the next stages. The results of this investigation are described in the last article of this thesis.

HYPERVITAMINOSIS A INDUCED TERATOGENESIS

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INTRODUCTION

Congenital defects have long been considered as bizarre curiosities only deserving display in anatomical museums. Persons surviving with gross malformations were put away in the wards of institutions, and even some of these people tried to make a living by exhibiting themselves at fairs. However, in the last decades several medical disciplines gradually became interested in the problems of patients with congenital disorders. Genetics discovered the chromosomal bases of some syndromes, surgery and pediatrics developed techniques for the treatment of several anomalies, obstetrics reduced the incidence of perinatal brain damage, and even prenatal diagnostic tests became available.

Embryology contributed descriptions and analyses of prenatal development, and the investigators tried to correlate the morphology of birth defects with the developmental processes that had been discovered. However, definitive conclusions were difficult to make. The finding that congenital malformations can be produced in mammals by various exogenous agents opened a new area of research. This experimental teratology concentrated on the morphogenesis of defects, interaction of exogenous agents with developmental processes, and problems of drug safety in pregnancy. It was found that vitamin A is teratogenic when administered in high doses, and in almost all organ systems malformations were produced by this substance. The reports published on this subject not only present the data on one of the most investigated teratogens, but also illustrate the evolution of experimental teratology in the past years. Therefore, we decided to present a review of these publications.

To present a systematic and conveniently arranged survey, the malformations recorded in the literature are arranged by organ system. Cleft palate is dealt with separately, and some chapters are devoted to the pathophysiological aspects of vitamin A teratogenesis.

The day of copulation is referred to as the first day (D. 1) of pregnancy. The data referring to this day as "day 0" have been converted, so that day 0 becomes D. 1.

We refer to Kalter and Warkany's and Cahen's reviews for previous discussions on the teratogenic effects of hypervitaminosis A.[1,2] The malformations of the nervous system induced by excess vitamin A are also described in a chapter of Kalter's book.[3] In a work dealing with congenital anomalies in humans, Warkany frequently demonstrates the morphological resemblance of induced malformations (e.g., by hypervitaminosis A) to congenital defects in man.[4]

The following nomenclature was used: vitamin A alcohol (synonyms: vitamin A_1 alcohol, retinol, axerophtol), vitamin A palmitate (esterized vitamin A_1 alcohol), vita-

min A acetate (esterized vitamin A_1 alcohol) and vitamin A acid (retinoic acid, tretinoin). The concentration of vitamin A in preparations is generally expressed in IU (international unit). In biological activity, 1 IU corresponds with 0.344 g pure crystalline all-trans vitamin A acetate. In most of the reviewed articles, the dosage is indicated per animal; in some of the papers, it is indicated per kilogram body weight.

A description of the chemistry and physiology of this vitamin is outside the scope of this review. For these aspects, we wish to refer to some other reviews.[5,7]

MALFORMATIONS OF THE NERVOUS SYSTEM

Exencephaly

This is a severe malformation of the brain. It develops as a result of absent closure of the rostral part of the neural tube. Initially, it is characterized by protrusion of well differentiated brain tissue. In term fetuses, the brain tissue is degenerated and has a hemorrhagic aspect.[8,9] In this final stage, the malformation is called anencephaly (abscence of brain). When Cohlan treated pregnant Wistar rats orally with 35,000 IU vitamin A on D. 2, 3, or 4 to 16, 52% of the surviving fetuses showed this anomaly.[10-12] Giroud and Martinet also induced this condition in rats by administration of about the same dose.[13] In females treated with 20,000 IU from D. 2 to 15, this anomaly was seen in 6% of the fetuses.[14] If 60,000 IU was administered in 3-day periods, D. 8 to 10 appeared to be the most susceptible period (53%).[15-17] In Swiss-Webster mice a dose of 3000 IU on D. 8 to 10 produced exencephaly in 28 to 59% of the offspring.[18-21] In a further report, it was shown that it occurred in mice also after single treatment on D. 6 (1%), D. 7 (21%), D. 8 (12%), or D. 11 (0.9%).[22] Langman and Welch treated Wistar rats with vitamin A on D. 9 to 11.[9] Using doses of 30,000 to 60,000 IU, they found, respectively, 13 to 26% malformations of the nervous system, particularly exencephaly. But in Swiss-Webster mice the treatment had much less effect on brain development. In these experiments, only 0 to 4% malformations occurred with 3000 to 7000 IU administered on different days between D. 8 and 10. In CF/1 mice, the highest incidence (13%) of exencephaly was produced by i.p. administration of 15,000 IU water-soluble vitamin A on D. 8.[23] Shenefelt carried out extensive research in golden hamsters.[24] The females were treated i.v., i.p., or orally with 7 to 116 mg/kg vitamin A acid at several times between D. 1 and 13. For litters with 50% fetal lethality, graphs were presented in which the incidence of the defects in surviving fetuses was plotted against the time of administration. For exencephaly, a maximum value of 58% of the surviving fetuses existed after treatment on D. 8. Also, in the golden hamster exencephaly was found in 15.7% of the offspring after oral administration of 20,000 IU on D. 8.[25] However, the malformation could be induced only sporadically in rabbit fetuses.[18,19,26]

Thus, it can be concluded that exencephaly is easily produced in several species. The most susceptible period is on D. 8 to 10 in the rat, D. 7 and 8 in the mouse, and D. 8 in the hamster. If the different developmental rate in the species is considered, it can be concluded that the most susceptible stage is just prior to the formation of the rostral neural walls.

Morphogenesis

The anatomy and morphogenesis of exencephaly have extensively been described in the rat[9,15-18,27-37] and the mouse.[18,20,38] Early stages of exencephaly were studied in the golden hamster,[25,39] the rat,[40-42] and the mouse.[43]

In these papers, the development of exencephaly is clearly demonstrated. On D. 11, the cephalic neural walls do not fuse dorsally, but expand laterally and remain contin-

uous with the surface ectoderm. Thus, the neural closure does not take place, the malformed brain is not covered with surface ectoderm, and the neuroepithelial matrix layer remains exposed to the amniotic cavity. Whereas the prosencephalon and mesencephalon stay open, the caudal part of the rhombencephalon is closed. Rathke's pouch, the otic vesicles, and the Gasserian ganglion are normal.

D. 12 — The cerebral vesicles continue to grow and become dorsally covered by the everted diencephalon. The primordium of the neurohypophysis is present.

D. 13 — The primitive cerebral cortex is differentiating into a three-layered structure, but the olfactory bulb has not yet developed. The ganglia of some cranial nerves can be distinguished.

D. 14 — Due to continuing growth, the telencephalic walls are now convoluted, and the malformed brain forms a big bulging mass on the skull base.

D. 15 — The olfactory bulb starts to develop.

D. 16 — At the junction of neuroepithelium and surface ectoderm, choroid plexus tissue appears. The cerebral flexures are much less marked than in the controls.

D. 17 — The rostral tips of the telencephalon are prominent above the nose, whereas the rest of the cerebral tissue is covered by the widely everted diencephalon and mesencephalon. Meanwhile, the histogenesis of the malformed brain goes on and cell layers and several fiber tracts can be observed.

D. 18 — Whereas the brain tissue is still intact, in the subneural mesenchyme capillaries are rupturing, resulting in small hemorrhages.

D. 19 — In the superficial parts of the diencephalon and mesencephalon, edema and cell degeneration begins.

D. 20 — All of the brain substance except the rhombencephalon is involved in a spreading process of degeneration.

D. 21 — Only some parts of the brain can still be recognized. The rest has been transformed into an irregular hemorrhagic mass. The amniotic fluid becomes contaminated with blood and cellular debris.

Thus, in a period of 10 days a malformed, protruding but well differentiated brain (exencephaly) develops and degenerates (anencephaly).

Auroux et al. examined exencephalic rat fetuses of D. 16 to 19 in great detail.[27] The anterior commissure was small and developed 1 day too late (D. 17). Its anterior part did not run horizontally but ventrolaterally, due to the abnormal shape of the telencephalon. The fornix developed 2 days too late (D. 18) and had an abnormal course after it had originated from the laterally situated hippocampal primordium. There were many nerve fibers in the telencephalon, and part of them formed the internal capsule. However, owing to the failure of closure, no corpus callosum formation took place. In this material, the closure defect extended rostrally to the anterior commisure.

Although it is generally accepted that vitamin A-induced exencephaly is caused by nonclosure of the rostral neural plate, there are different hypotheses about the underlying mechanisms. Electron microscopic investigations of D. 9 rat embryos soon after administration of excess vitamin A revealed abnormalities of the intracellular space, especially in the mesoderm.[41] Small defects consisting of shrinkage of the cells with accompanying enlargement of intercellular space and widening of subneural blood vessels[44] were observed 6 hr after administration in the cephalic mesoderm. This became more severe 8 to 10 hr after treatment, and at this stage, there were even necrotic mesoderm cells. Because of this degenerative process, the cephalic mesoderm collapsed. This caused a flattening of the neuroepithelial folds and thus interfered with the neural tube closure.[42-44] So this first hypothesis is based on the observation that vitamin treatment produces abnormalities of the mesoderm, which subsequently result in exencephaly.

Underdevelopment of the cephalic notochord has also been suggested as the cause of exencephaly [17 30 31] However, on D 16 to 20, a normal development of the notochord was found in exencephalic fetuses [45 47]

Another hypothesis was proposed by Langman and Welch [9] They observed excessive mitosis in the matrix layer in exencephalic fetuses of D 13 to 15 As a consequence, the internal limiting membrane seemed locally ruptured, and cytoplasm vesicles protruded from the surface of the everted diencephalon The resulting loss of cellular contacts was considered as the cause of absent closure

A fourth hypothesis suggested that excessive proliferation of the neuroepithelium in a lateral direction was responsible for the development of exencephaly [28]

When comparing the volume of amniotic fluid of fetuses with exencephaly and fetuses with a normal brain, the amount of amniotic fluid was ten times as much in the exencephalic fetuses.[48 49] It was suggested that due to the extroversion of neural tissue and choroid plexus, cerebrospinal fluid (CSF) was secreted into the amniotic cavity The amniotic fluid in exencephalic fetuses was sanguinolent as a result of hemorrhages in the degenerating brain tissue, and it contained an increased amount of fetal proteins [30 51 53] Therefore, it can also be assumed that the hydramion was not only caused by leakage of CSF, but also by exsudation of fetal serum

Meningocele and Meningoencephalocele

Several authors described encephaloceles in rat fetuses after administration of 50,000 IU vitamin A on D. 8 to 10 [30 54 58] Small parts of the brain protruded from the skull near the vertex In the protruding cerebral cortex, heterotropic cell nests were occasionally found In some fetuses, the mesencephalon was not completely closed dorsally, and in others, only meningeal tissue protruded Here the osseous cranial vault was missing, and the epidermis consisted of two or three cell layers with slight keratinization but without hair follicles. In these cases, epithelial tubuli with an occasional neural cell were sometimes found between epidermis and brain Giroud considered those meningoceles and meningoencephaloceles as minimal failures in the process of neural tube closure [30] Similar malformations were described in rat fetuses after administration of 50,000 to 100,000 IU on D 8 to 10 of gestation [59] Encephalocele was found in mice after administration of 15,000 IU vitamin A on D. 10.[23] Shenefelt mentioned that the incidence of encephalocele in hamster fetuses was highest (17%) after administration on D 8[24] Neonatal rats which had a meningocele had an opening at the vertex of the skull covered with thick dura tissue[12] 30 days after birth These animals did not show any deviating behavior

In summary, it can be concluded that meningocele and meningoencephalocele are minor closure defects of the neural tube. They can be induced by teratogenic treatment prior to neural tube closure.

Spina Bifida

Lumbar spina bifida aperta characterized by an unclosed neural tube occurred in rat fetuses in low frequency (3%). The spinal ganglia were normally developed, and in some cases, degeneration of the neural tissue was found [15 17 33 54 55] Marin-Padilla and Ferm found in some cases local hyperplasia of the notochord in the region of a sacral spina bifida aperta.[25] In 12% of the hamster fetuses, spina bifida occurred after maternal treatment on D 8 with 20,000 IU vitamin A Spina bifida aperta and occulta in almost all of the surviving golden hamster fetuses was found after administration of vitamin A acid on D 8 or D 9 [24]

It is remarkable that excess vitamin A very frequently produced a closure defect of

the brain (exencephaly), but only seldom abnormal closure of the spinal cord (spina bifida).

Hydrocephalus

Benito-Arranz described hydrocephalus in 20% of the fetal rats after maternal treatment with 40,000 IU vitamin A on D. 1 to 10.[60,61] Also, in litters of rats that had been given 35,000 IU vitamin A on D. 2 or 4 to 16, hydrocephalic fetuses characterized by enlarged dome-shaped cranial vaults were found.[12] Murakami and Kameyama also found hydrocelphalus (2.8%) in mice after i.p. treatment on D. 10 with 15,000 IU vitamin A.[23] Thus, hydrocephalus does not occur frequently after vitamin A treatment. Therefore, it is not feasible to determine the most susceptible period or the morphogenesis. Only in one experiment were some data obtained to clarify the pathogenesis of this malformation. In litters of females treated orally with 10,000 IU vitamin A on one of D. 9 to 13, some fetuses showed narrowing or obliteration of the cranial cisternae and Sylvian aquaduct.[3,62] Although there was no hydrocephalus at that moment, it would probably have developed in the post-natal period. This indicates that a CSF circulation disturbance accounts for the development of vitamin A-induced hydrocephalus.

Microcephaly

In combination with a number of other malformations, microcephaly was induced in A/Jax mice with 2 mg vitamin A acid on D. 9 or 20,000 IU vitamin A acetate on D. 9, 10, or 11 of gestation.[63] In CF/1 mouse fetuses, the highest incidence (52%) of this malformation was induced on D. 9.[23]

In 100% of the surviving fetuses, microcephaly occurred after administering vitamin A acid on D. 9.[24]

Cyclocephaly

Cyclocephaly was described in the mouse after administration of 500 IU on D. 8 to 10.[64-67] The head was reduced in size, the telencephalon had only one ventricle, and olfactory bulbs were missing. The fila olfactoria formed a bulb-shaped mass on the cribriform plate without reaching the brain. The cerebral cortex was almost normally developed. From the diencephalic region, a median optic stalk had evaginated, and only ocular rudiments, partially fused in the midline, were detected. The telencephalic and diencephalic ventricles communicated via one median foramen. The pineal gland was duplicated in some cases, and the hypophysis was lacking. It was supposed that this malformation was caused by a vitamin A-induced lesion of the prochordal plate.

Other Brain Abnormalities

Duplication of the hypophysis, hypothalamic infundibulum, and cranial and cervical notochord occurred in a rat fetus whose mother had been treated with excess vitamin A during gestation.[68,69]

Some investigators used vitamin A-induced exencephaly as a model for fetal endocrinological investigations.[70-72] The abnormal fetuses did have an anterior hypophyseal lobe, but no hypothalamus. The thyroid gland was normal as far as weight and histology were concerned.

From further experiments, it was concluded that in D. 16 to 22 exencephalic fetuses, the hypophysis persisted but was pulled upward and had a triangular shape.[73] It appeared that the adrenal growth continued normally until D.20, but on D. 20 to 22 shrinkage and vacuolization of the adrenocortical cells and decrease of adrenal weight took place.

OCULAR MALFORMATIONS

When 20,000 IU vitamin A was administered to pregnant Wistar rats on D. 2 to 16, 25% of the fetuses showed anophthalmia, microphthalmia, exophthalmos, or cataract.[11,14,29,32] In later experiments, it appeared that the incidence of ocular abnormalities varied with the time of treatment.[15-17] With administration of 60,000 IU on D. 5 to 7, only 4% anophthalmia and microphthalmia were found, but treatment on D. 8 to 10 or D. 10 to 12 produced these anomalies in 73 or 30% of the fetuses, respectively. Palpebral aplasia was observed in 5% (D. 10 to 12) or 28% (D. 11 to 13), and cataract in 9% (D. 18 to 20). In addition to the determination of the susceptible periods, descriptions of these anomalies were presented.[33] In anophthalmia, only palpebrae, conjunctiva, external muscles, and lacrimal glands were present. In microphthalmia, the retina rudiment consisted of only two epithelial layers, and differentiation of lens fibers seldom occurred. With exophthalmos, the dimensions of the bulb were normal, but the orbit was too shallow. In cases of cataract, almost all lens fibers had degenerated, but the lens epithelium was intact. Exophthalmos and palpebral aplasia could also be induced in mice.[20-22] Sometimes these abnormalities of the eye were combined with facial clefts.[74]

Malformations of the eye and their morphogenesis were described in several papers.[23-25,75-80] In these investigations, different species, different doses, and various times of treatment were used. However, some general conclusions can be drawn. Anophthalmia is induced in a rather early stage in which the optic evagination just starts to develop. When the optic evagination and optic cup have already developed, teratogenic treatment induces microphthalmia. In approximately the same period, the choroid fissure is closing and interference with this process might lead to coloboma retinae. Exophthalmos is characterized by a normal ocular bulb which protrudes due to underdevelopment of the orbit. Thus, this malformation is produced when the eye has already developed, but the orbit still has to reach its final shape. Congenital cataract is induced by treatment late in fetal life. This is a result of degeneration of the lens fibers, which form relatively late in gestation.

A typical ocular anomaly was induced in the rabbit after administration of 125,000 IU vitamin A on D. 5 to 10.[81] Six fetuses of one litter had big protruding red eyes with coloboma. Fibrosis of the primitive corpus vitreum appeared to attract the posterior pole of the eyeball, causing rupture of vessels and hemorrhages, which pervaded into the primitive lens. In a later stage, retinal hematomas with ablatio retinae also developed. In the rat, another anomaly was seen.[82-84] The nerve fibers ran between the retinal cell layers to accumulate close to the malformed iris. As a consequence, no optic nerve was formed.

With cyclocephaly in the mouse, there were two hardly separated ocular rudiments.[65,82,83] In one of them, there were some nerve fibers that did not reach the diencephalon, but there was an optic stalk. The other one had no optic stalk, and the rudimentary retina was incorporated in the diencephalic wall. From this retina, the nerve fibers grew in rostral direction.

A peculiar case of eye malformation in a mouse fetus was produced by oral administration of 40,000 IU vitamin A on D. 9.[85] The left eye was small and had no optic nerve. The right eye was transformed into a vesicular structure consisting of a thick retina. Lens, iris, cornea, anterior chamber, and ciliary body were lacking. The author supposed this to be a case of excessive coloboma with retinal overgrowth causing extrusion of the lens. In all other histologically examined fetuses, coloboma was found.

In some species not commonly in use for teratological experiments, eye malformations were also induced. Palpebral aplasia was observed in fetuses of *Macaca nemestrina* and rosettes and folds in the regina and cataract were observed in pigs after treatment with excess vitamin A.[86-88]

MALFORMATIONS OF THE EAR

Several investigators described malformations of the ear in rat fetuses as a result of excess vitamin A during pregnancy.[89-92] There was a narrowing of the membranous labyrinth, and there were too few spiral turns in the cochlea. The space between cochlea and vestibula was narrowed. The organ of Corti, the spiral ganglion, and the vestibulocochlear nerve were underdeveloped. There were also anomalies of the semicircular canals. Preauricular appendages were noted in *Macaca nemestrina* fetuses after oral treatment with 10 mg/kg vitamin A acid on D. 21 to 45 of gestation.[86,87] In Giroud and Martinet's material, some rat fetuses had auricular aplasia and preauricular appendages consisting of cartilage covered with skin.[15-17,33,93] The hypoplastic tympanic cavity did not contain auditory ossicles. The Eustachian tube ended blindly medially or was absent. Synotia combined with microstomia occurred in the rabbit.[18] Hayashi induced several malformations of the auricula in ddN mouse fetuses by injection of 15,000 IU on D. 9.[94] These anomalies could be subdivided into low-set auricles, hypoplasia, or aplasia. The severity of the malformations of the external auditory meatus, middle, and inner ear was reflected in the degree of malformation of the auricle. Other papers described aplasia, malformation, or low-set auricle in 58% of the fetuses.[75,95,96] The Eustachian tube was often atretic, and ectopic thymus tissue was located in or near it. In these experiments, the pregnant mice had been treated orally with 10,000 IU vitamin A on D. 9 and 10. In *Cavia*, aplasia or malformation of the auricula, hypoplasia or aplasia of the bulla tympanica, and synotia combined with agnathia was found after treatment with 200,000 IU on D. 15, 16, or 17.[77] Anomalies of the auricle (aplasia, malformation, low setting) occurred in golden hamster fetuses with a maximum incidence (100%) after treatment 7¾ or 8 days p.c.[24]

CRANIOFACIAL MALFORMATIONS

When examining a great number of mouse fetuses, after maternal treatment with 10,000 IU vitamin A on D. 9 or 10, Kalter and Kalter and Warkany noted perioral tags of skin tubercles (62.5%), microstomia with proximal shortening of the mandible (32.6%), distal shortening of the mandible (17.8%), shortening of the upper jaw (11.2%), median cleft mandible (2.0%), and underdeveloped zygoma with exophthalmos.[95-98] The incidence of cleft palate (70.8%) was, in fact, higher because only specimens without severe oral malformations were examined for cleft palate. Histological examination revealed a narrow oral cavity with a low positions of the palatal processes, which were fused with the lateral oral epithelium. The malformed tongue was fused with palate and gingiva. The teeth were fused, ectopic, or aplastic. The ramus mandibulae was almost completely lacking, the corpus mandibulae was malformed, and Meckel's cartilage was missing. The severely malformed maxilla formed an ankylosis with the corpus mandibulae. The maxillar zygomatic process came from this area and was surrounded by ectopic fragments of cartilage. The lateral parts of the nasal cartilage were directed ventrolaterally instead of ventromedially. The sublingual and submaxillary musculature[1] was disorganized or aplastic. The musculus[2] masseter, parotid duct, and sublingual salivary glands and ducts were missing, and the submaxillary glands were present unilaterally only. In addition to these malformations, other inves-

tigators found astomia, zygoma aplasia accompanied by aplasia of the musculus masseter and exophthalmos, and complete or partial aplasia of the mandibular joint.[75]

Also, in fetuses of mice treated i.p. with 15,000 IU on D. 8 or 9, proximal mandibular hypoplasia with short and malformed Meckel's cartilage was induced.[99] When the teratogen was administered on D. 10 or 11, the mandibular hypoplasia was accompanied by increased thickness, particularly in the intermediate and the distal parts. Ectopic maxillar cartilage was formed after the development of normal cartilage on D. 14 or 15. Deuschle et al. described some rat fetuses with extreme bilateral or unilateral exophthalmos.[100] However, in addition, there were malformations of the nasal cavity, posterior cleft palate, and ectopic cartilage between the palatal shelves, maxilla, and mandibula. The proximal part of the mandibula was shortened. The zygomatic process and the maxillar alveolar process were often missing, and the molars were absent or underdeveloped.

Moreover, the dose-response relationship was examined for the oculofacial syndrome in the mouse (exophthalmos, microcephaly, micrognathy, microstomia, microtia), in the rat (identical malformations with median cleft mandible), and the rabbit (palpebral agenesis, microcephaly, maxillar shortening).[101] This relation was most distinct in the rat, next in the mouse, and then rabbit. The minimum dose (50,000 to 75,000 iu/kg) to induce these abnormalities was almost similar for the three species, when an i.p. injection was given on D. 8, 9, or 10 in the mouse, D. 10 or 11 in the rat, and D. 9 or 10 in the rabbit. Retarded development of the maxillar process with ectopic cartilage development and dental agenesis occurred in the rat after treatment with 100,000 IU on D. 10 or D. 10 and 11.[102] The cartilaginous nasal septum was reduced in height. As a whole, however, the nasal septum was not. The mandibula was less thick than normally, and the tongue was broadened. Skull base malformations and their development in exencephaly were described in median sections.[45-47] There was an increase in the angle between the cervical vertebral column and the basioccipital bone and the reduction of the angles between basioccipital and basisphenoid, and basisphenoid and presphenoid. The anomalies resulted in shortening of the skull base. In exencephalic fetuses, an abnormal angle between maxilla and basisphenoid also existed. Moreover, aplasia of the os tympanicum, hypoplasia of the temporal squama, and microstomia occurred.[40] In a paper on vitamin A-induced congenital dental malformations, Knudsen also described maxillomandibular ankylosis and agenesis or hypoplasia of the mandibular joint, muscles of mastication, mylohyoid, and digastric muscles in the rat fetus.[103] Murakami and Kameyama gave CF/1 mice a single i.p. injection of 15,000 IU vitamin A on 1 day between D. 8 to 13.[23] The cranial malformations observed were micrognathy (maximum after treatment on D. 9: 57%) and malformed or absent mandibula (maximum D. 9: 82%). In the golden hamster, Shenefelt induced narrowed or single nasal cavities (maximal incidence: 10% after treatment 6¼ days p.c., cleft palate: 88% 9¼ days p.c., cleft lip (unilateral or bilateral): 64% 8¾ days p.c., cleft mandible, maxillomandibular ankylosis with ectopic cartilage, and malformation of masticatory musculature, microstomia, epithelial fusions in the mouth, tongue hypoplasia, cleft tongue, fusion of the tongue with the lateral oral epithelium, aplasia of the incisors or supernumerary incisors, aplasia or fusion of the molars, hypoplastic submaxillar gland, and aplasia of bones of the skull base.[24] Experiments with vitamin A acid in Rhesus monkeys produced one fetus with a facial anomaly.[104] Abnormal shape of the skull was also noted in fetuses of *Macaca nemestrina*.[87]

Nasal clefts were observed only occasionally in mouse fetuses.[74] In rabbit fetuses, excess vitamin A induced mandibular hypoplasia and narrowing of the skull, which resulted in a typical bird-like appearance.[18,19,26]

From these papers it can be concluded that a great variety of craniofacial malfor-

mations can be induced. The most susceptible period appears to be D. 8 to 10. Ectopic cartilage is produced by treatment in a slightly older embryonic stage.

Malformations, aplasia, abnormal localization of incisors and molars, and hyperdentition have already been mentioned, but the following publications mainly referred to dental anomalies. In Knudsen's extensive publications, anomalous dentition in rat fetuses, induced by vitamin A overdosing during gestation, was described in detail.[103 105-112] The study of the morphogenesis of hypoplastic teeth in the mouse suggested that hemorrhages disorganized the tooth bud which resulted in dental hypoplasia.[113 114] Masi induced a case of cyclocephaly by treating pregnant mice with 1000 IU vitamin A on D. 8 to 10.[115] In this case, only one upper incisor was observed. It was located in the median part of the upper jaw.

CLEFT PALATE

Cleft palate morphogenesis was studied in the rat after administration of 60,000 IU vitamin A on D. 10 to 12 or D. 11 to 13.[116-119] This treatment caused the malformation in about 80% of the fetuses. On D. 16, the vertical palatal shelves were small or absent. On D. 17, they were rotating to the horizontal plane, and on D. 18, the small malformed shelves were horizontal but not fused. Lotosh found that cleft palate caused by treatment in the most susceptible period (D. 11 or 12) was characterized by a vertical position of the palatal shelves.[120-123] However, after treatment in later stages, the unfused shelves were rotated to a more horizontal position.

The mechanism of this abnormal process was investigated with ^{3}H-thymidine.[124] In this autoradiographic investigation, a reduced epithelial proliferation in the posterior parts of the palatine shelves was found. Experiments with ^{35}S labeling demonstrated an increased incorporation of ^{35}S in the palatal shelves, nasal rudiment, ectopic cartilage in the maxillar region, and limbs.[116 117] Scintillation-counting in homogenized mouse fetuses revealed that the ^{35}S uptake was considerably increased 24 hr after vitamin A administration.[125] However, based on experiments demonstrating elevated ^{35}S uptake in hypervitaminosis A-induced cleft palate and reduced uptake in cortisone-induced cleft palate, it was pointed out that no relation between altered mucopolysaccharide metabolism and cleft palate development could be proved.[119 126 127]

Ross and Walker displaced the embryonic tongue from its position between the palatal shelves in a ventral direction.[128] Afterwards the growth of the shelves was measured in control and vitamin A-treated fetuses. In the treated ones, growth of the shelves was diminished. Thus, the development of the palatal shelf itself was disturbed, and interposition of the tongue appeared not be be the primary cause of cleft palate formation in these experiments. Accurate measurements in vitamin A-induced cleft palate showed that this malformation was not related with the associated mandibular growth retardation.[129 130]

In in vitro experiments, vitamin A added to the medium caused growth retardation and cleft palate in explanted palatal shelves.[131] Fusion could occur in vitro even though before explantation of the shelves, the pregnant female was treated with teratogenic doses of vitamin A. Hence, it was concluded that the lateral skull growth also plays a role in the development of cleft palate.

The highest incidence of this malformation in the rat (92%) occurred after administration on D. 11 to 14.[15-17] Both on D. 9 and 11, the susceptibility for cleft palate induction in mice was greatest.[22] In the mouse, the maximal incidence (90%) of cleft palate occurred after i.p. injection of 10,000 IU on D. 10.[113] Kochhar found a peak incidence in the DBA/2J mouse after treatment with vitamin A acid on D. 10 (68%) and D. 12 (95%).[132] After administration on D. 10, cleft palate seemed to be secondary

to the embryonic growth retardation and associated orofacial malformations, but after D. 12 the treatment primarily seemed to affect the palatal shelves. In CF_1 mouse fetuses, the maximum incidence of cleft palate (74%) was induced with i.p. injection of 15,000 IU on D. 11.[23] In fetuses of *Macaca nemestrina*, cleft palate was found after maternal treatment with 10 mg/kg vitamin A acid on D. 21 to 45.[86,87]

With cleft palate induced by hypervitaminosis A, the palatine arteries also showed an abnormal growth pattern.[133,134]

The results of experiments described in several other papers are grossly similar with those from articles mentioned above.[135-146]

DEFECTS OF THE CIRCULATORY SYSTEM

Giroud and Martinet produced a case of ventricular septal defect in a mouse fetus by administration of 3000 IU on D. 8 to 10.[19] Other authors observed vitamin A-induced ventricular septal defect, overriding aorta, narrow ductus arteriosus, and transposition of the great vessels in mice fetuses.[96,97] In some cases, the aorta and the pulmonary artery originated from the right ventricle, and occasionally a coronary aneurysm was present. In fetuses with umbilical artery agenesis, an aberrant superior mesenteric artery served as a substitute. Transposition of the great vessels was also induced in a *Macaca nemestrina* fetus.[87] Palludan treated a pregnant pig with 3,000,000 IU vitamin A every other day from D. 12 to 42.[88] Three of the ten fetuses had a ventricular septal defect. When a female was administered 10,000,000 IU vitamin A from D. 16 to 19, after delivery one of the young pigs grew slowly. Autopsy revealed stenosis of the aorta, malformation of the bicuspidal valve, pulmonary edema and hypertrophy, and dilatation of the pulmonary artery. When Robens administered 400,000 IU/kg to golden hamsters on 1 day between D. 7 to 11, the malformed and very small fetuses survived in an incubator only for a short period of time.[77] Postmortem examination showed underdevelopment of the left atrium, ventricle, and truncus arteriosus. In experiments with golden hamsters, ventricular septal defect, transposition of the great vessels, aortahypoplasia, and the presence of one ventricle were found.[24]

DEFECTS OF THE RESPIRATORY SYSTEM

Vitamin A-induced malformations of the lungs were described in the golden hamster.[24] The normal pattern of three right lobes and one left lobe was inversed, or there was only one lobe per lung or complete or partial aplasia of the right caudal lobe.

DEFECTS OF THE DIGESTIVE TRACT

In the literature, many digestive tract anomalies are described in relation with the syndrome of caudal regression (see section entitled Skeletal Defects).

Anal atresia occurred in a *Macaca nemestrina* fetus as a result of maternal treatment with vitamin A acid.[87] Umbilical hernia was found by Kochhar in the rat.[63] Murakami and Kameyama noted anal atresia and umbilical hernia in CF/1 mice.[23] A rectourethral fistula was observed in a pig after oral maternal treatment with 10,000,000 IU from D. 16 to 19.[88] Robens mentioned anal atresia in *Cavia*,[77] and situs inversus, umbilical hernia, and agenesis of the bile bladder was induced in the golden hamster.[24]

UROGENITAL DEFECTS

Hydroureter and Hydronephrosis

This mostly bilateral defect was found in rat fetuses, with a peak incidence of 40% after administration of 50,000 IU vitamin A on D. 8 to 10.[30 147-149] The glomeruli were intact, but the renal pelvis was much widened, and the papillae were not prominent. The collecting tubules was also dilated. In 64% of the cases, the ureter was extremely wide and long. Its urinary bladder was often underdeveloped and occasionally not even distinguishable from the cranial part of the urethra. The orifices of the ureters frequently ended in the ejaculatory duct, urethra, prostate, or vagina. Apparently, the orificium ureteri had not migrated from the Wolffian duct to the urogenital sinus. In a number of cases, atresia of an ureter was found. In other fetuses, a vesicoureteral membrane (consisting of epithelium of the urogenital sinus) persisted to D. 20 or 21, instead of to D. 19. Since the kidney produces urine as early as D. 19, the distal atresia can be considered the cause of the hydronephrosis and hydroureter.

Baba and Tsuruhara and Tsuruhara gave 250,000 IU/kg to rats throughout pregnancy.[150 151] On D. 16 and 17, no renal defects were found, but after the glomeruli had developed on D. 18, progressive dilatation of the renal pelvis was observed. Since no stenosis or obstruction in the urinary tract was observed, it was concluded that there had to be a functional disorder of this system.

Hydronephrosis and hydroureter were also observed in mice[96 97 152] and golden hamsters.[24 76 77]

Other Defects of the Urinary Tract

A great number of urinary tract defects have been induced: bilateral renal or ureter agenesis, ectopia renis, fused kidneys with one or two ureters, anastomosis, atresia or duplication of ureters, ectopic orofice of the ureter in the bladder, diverticles, and dilatation of the bladder.[96 97 152] Blind-ending dilatated ureters and polycystic kidney were seen in fetuses of *Macaca nemestrina* after vitamin A administration.[87]

Genital Defects

In mouse fetuses, bilateral agenesis of the Müllerian duct, fusion of the Müllerian and Wolffian duct, and atresia of the Wolffian duct were induced. Sometimes a Wolffian duct ended contralaterally in the urinary bladder.[152] Uterus unicornis was present in the *Cavia* after maternal treatment with 200,000 IU/kg on D. 16.[77] Shenefelt described cryptorchism, duplication of the testes, hypoplasia of the uterus, and hypoplastic or aplastic genital papilla.[24]

SKELETAL MALFORMATIONS

Extremities

Love and Vickers found severe limb deformities in 70% of the fetuses after administering 200,000 to 800,000 IU/kg vitamin A to Sprague-Dawley rats on D. 11 to 17.[153] The defects were equally frequent on the right and left side, in the fore as well as in the hind limbs. However, those of the forelimbs were more severe than those of the hind limbs. The observed malformations were phalangeal aplasia, hypoplasia, syndactyly or deformity, and shortened long bones. The deformities seemed to be caused by a mesodermal disturbance and a consequent effect on the enchondral and periostal ossification.

Another study compared deformities which were induced in the CF/1 mouse, Sprague-Dawley rat, and the rabbit with single doses of 600,000, 500,000, and 300,000 IU/

kg,[154] respectively. Micromelia occurred in the mouse after treatment on D. 10 and 11 and the rat after administration on D. 11 and D. 12, but it was not observed in the rabbit. The incidence in the forelimbs was mostly higher and could be induced one day earlier than in the hind limbs. This malformation was characterized by aplasia of fibula or ulna and by shortening, curvature, or thickening of the remaining bones. The most susceptible periods for the induction of oligodactyly, brachydactyly, or syndactyly were D. 9 to 12 in the mouse, D. 10 to 14 in the rat, and D. 10 to 13 in the rabbit. The incidence was highest in the mouse, then in the rat and the rabbit. Dose-response experiments with 50,000 to 250,000 IU/kg in the mouse and rabbit on D. 10 and in the rat on D. 11 showed a distinct dose-response relation for micromelia and digital malformations.

In the DBA/2J mouse, a certain temporal order was demonstrated in which the different bones of the limbs at first were susceptible and later became resistant to the teratogenic action of vitamin A acid.[132] Also, a rostrocaudal and a proximodistal gradient were demonstrated. The highest incidence of defects was induced by treatment on D. 11 to 13. It was concluded that vitamin A not only affects the mesenchymal condensation in the limb bud, but also interferes with futher stages of differentiation. In other papers, a great variety of malformations were described in mouse fetuses: clubfoot, aplasia, hypoplasia or ectopia of the hallux, micromelia, agenesis or hypoplasia of the distal part of the forelimbs, abduction of the radius, oligodactyly and syndactyly, and agenesis, thickening, shortening, or curvature of the long bones.[95,96]

By means of electron microscopy, abnormalities were observed as early as 4 to 24 hr after treatment.[155-157] In short, the defects consisted of swelling of membranes, cellular shrinkage, and degeneration with enlargement of the intercellular space. Possibly there was some repair of affected tissue.

When the limb bud was studied histologically after 24 hr after treatment on D. 11, the shape was pointed instead of discoid.[158,159] After 48 hr there was still no sign of digital development, and many necrotic cells were present, but after 72 hr the number of necrotic cells had decreased. There was no preaxial mesenchymal condensation. The interdigital grooves were too shallow, but their number already showed that oligodactyly would develop.

Biochemical and electron microscopical analysis of fetal rat limbs 4 days after maternal treatment with 100,000 IU on D. 12 revealed an increased sulfated glycosaminoglycan turnover, destruction of chondrocytes, increase of collagen fibers, and reduction of proteoglycan granules.[160] In later stages, the perichondrial vascularization was insufficient.

Other Skeletal Defects

Murakami and Kameyama injected 15,000 IU vitamin A i.p. in pregnant CF/1 mice.[23] After injection on D. 8, 80% of the fetuses had caudal aplasia or hypoplasia. After injection on D. 9 to 12, the incidence sharply decreased. Thus, in a very early stage in which mesoderm formation in the caudal region is not yet completed, the teratogen has the most substantial influence on tail formation. Malformations of lumbar and cervical vertebrae, pelvis, shoulder girdle, and ribs were also present.

Fusion and malformations of ribs and sternum, missing vertebrae, and defects of scapula and pelvis were induced in mice,[63,95,96] hamsters,[24,25] and *Cavia*.[77] Fantel et al. induced kyphosis, kyphoscoliosis, and tail abnormalities in *Macaca nemestrina* fetuses.[86,87]

Syndrome of Caudal Regression

Roux and Martinet described a syndrome in mouse fetuses after administration of excess vitamin A on D. 7 to 9 or D. 9.[161] In animals with caudal aplasia or hypoplasia, they found a pattern of malformations referred to as the syndrome of caudal regression. It was characterized by malformations of the urinary tract: horseshoe kidney, kidneys situated closely together, hydroureter with hydronephrosis, atresia of the ostium ureteris, dilatation of the bladder and atresia of the urethra, and ejaculatory duct ending in the urinary bladder; defects of the digestive tract: caudally blind-ending colon, colon ending in the urinary bladder, rectal aplasia, and atresia; malformations of the lumbosacral vetebral column; and duplication of the spinal cord or spina bifida with meningocele.

ABNORMAL POST-NATAL DEVELOPMENT

After maternal treatment with 10,000 IU vitamin A on D. 16 to 18, morphological defects in the cerebral cortex associated with spasticity, tremors, or hyperactivity were observed in neonatal Swiss-Webster mice,[162] and ataxia was described in neonatal *Cavia* after treatment with 200,000 IU on D. 15, 16, or 17.[77] Malakhovskii and Prozorovskii and Malakhovskii treated pregnant rats with 150,000 IU vitamin A on D. 9.[163-165] The offspring appeared normal, but when they were tested 1 month post-natally, they showed hypoactivity and reduced avoidance acquisition. However, no histological defects were observed in the brains. The offspring of rats treated with excess vitamin A on D. 9 to 11 had swimming maze deficits, but they exhibited no morphological defects of the brain.[166] Another publication demonstrated that offspring of rats treated with 60,000 IU vitamin A on D. 14 and 15 had decreased response inhibition.[167] Although some animals showed microcephaly or enlarged ventricles, a relation between brain morphology and reduced acquisition could not be demonstrated. When 90,000 IU vitamin A was administered on D. 17 and 18, the young rats had a slower rate of response in discrimination training, but they showed no brain abnormalities.[168] Vorhees examined the offspring of rats treated with 100,000, 40,000, 25,000, or 10,000 IU/kg from D. 8 to 10.[169] Hypoactivity was observed in animals of the 100,000 IU group, but they were handicapped by an unsteady gait with straightened hind limbs. In all groups, avoidance and discrimination acquisition were reduced. In a study of the physical development of young rats from females treated with 100 to 300,000 IU/kg daily during gestation, reduced food consumption, growth retardation, and poorly developed fur were found, and at 4 months, the animals were only half as big as the controls.[190]

So it can be concluded that even when gross defects are absent at birth, minor brain defects or growth disturbances can become manifest in post-natal life.

TERATOGENIC EFFECTS IN DIFFERENT SPECIES

Several species (mouse, rat, *Cavia*, hamster, rabbit, dog, pig, chick, monkey) are susceptible to the teratogenic action of vitamin A, but few comparative investigations were made on the differential susceptibility.

Kalter and Warkany found no difference in type and frequency of anomalies in A/J, DBA/1J, and C_3H/J mice.[96] Also, no differences in severity and incidence of limb deformities were noted in ddN and CF/1 mice. However, the most susceptible phase did differ: ddN, D. 12 and 13 and CF/1, D. 11 and 12.[171,172]

Other investigators compared the incidence of several malformations in mouse (CF/1), rat (Sprague-Dawley), and rabbit after treatment in equivalent developmental

stages.[154] The doses had been determined on the basis of maternal subacute toxicity tests and fetal lethal dose. For the mouse, the administered dose was 600,000 IU/kg on 1 day between D. 8 and 13; for the rabbit, 300,000 IU/kg (D. 8 to 16); and for the rat, 500,000 IU/kg (D. 10 to 15). The incidence of resorption and fetal lethality decreased in the order: rabbit, mouse, rat. For micromelia, the order was: mouse, rat (rabbit did not show micromelia). For digital malformations: mouse, rat, rabbit, and for oculofacial malformations: rat, mouse, rabbit. When three strains of rats (Wistar, conventional Sprague-Dawley, cesarean-derived Sprague-Dawley) were compared, marked differences were also noted in susceptibility and in distribution of the various defects.[173] Ohmori compared induced rib malformations in ddN/JCL and CF/JCL mice by injecting the females with 15,000 IU on 1 day between D. 7 and 12. After treatment on D. 10, lumbar ribs occurred in normal frequency in ddN/JCL and in increased frequency in CF/JCL fetuses, but in the former strain, the incidence of cervical and lumbar ribs increased after D. 11 treatment.[174]

MINIMUM EFFECTIVE DOSE

Most teratologists used vitamin A in extremely high doses, and considerably less attention was paid to the lowest embryotoxic dose in the varous species.

Some experiments showed that in Swiss-Webster mice, 6250 IU/kg, administered on D. 8 to 10, induced a certain percentage of resorptions and malformations.[8,175] After reduction of the dose to 2500 IU/kg, there were still a number of resorptions, but not of malformations. Thus, relatively low doses, approaching the human therapeutic dose (±5000 IU/kg), were embryotoxic in the mouse. For the Wistar rat, it was determined that 300,000 IU/kg caused less than 1% malformations after treatment on D. 8.[176]

It must be concluded that the data concerning the threshold for the induction of embryotoxic effects by vitamin A are scarce and diverse. For comparison, the daily requirements of vitamin A in experimental animals are presented: mouse, 1 to 2 IU; rat, 60 IU; golden hamster, 100 IU; *Cavia*, unknown;[177] rabbit, unknown;[178] and beagle dog, 45 IU.[179] For man, 20 IU/kg body weight a day was indicated.[180]

INTERACTION WITH OTHER AGENTS

Combined treatment with excess vitamin A and several other agents has been done in many experiments. The aim of this research mainly was to get a better insight in the teratogenic mechanism of vitamin A.

Cortisone enhanced the teratogenic effects of hypervitaminosis A.[119,121,126,181,185] Dexamethasone also showed this effect.[186] However, in other experiments, cortisone or adrenectomy had no influence on the embryotoxicity of vitamin A.[187]

The hypothesis that the thyroid is involved in teratogenesis was tested by combined administration of excess vitamin A with methimazole or methylthiouracil[121,185,188,190] or by supplementation with thyroxin.[121,185] Induced hypothyroidism appeared to reinforce the embryotoxicity of vitamin A, whereas thyroxin had a limited effect.

A possible relationship with carbohydrate metabolism was investigated by simultaneous administration of excess vitamin A and the hypoglycemia-inducing drugs, insulin,[182,185] propamide, or tolbutamide.[186] With this treatment, the incidence of malformations was reduced in comparison with treatment with vitamin A alone. However, Cohlan and Stone did not find any effect of insulin on the vitamin A teratogenicity.[187] Agents which supposedly cause hyperglycemia (dinitrophenol and dexamethasone) increased the effect of vitamin A,[186] but alloxan showed no effects.[185]

Several vitamins have been tested for a possible influence on vitamin A-induced

teratogenesis. Vitamin B complex,[185 191] vitamin B_6, folic acid,[192] vitamin C, vitamin D,[121] and vitamin E[193] had a protective effect. However, in another report, the protective influence of vitamin C was not confirmed.[185] In low doses, vitamin B_{12} reduced the vitamin A embryotoxicity, but in higher doses, it slightly enhanced this action.[194] No change in teratogenicity was observed by combining vitamin A with vitamin B_2.[192] Vitamin B_1 deficiency increased the embryotoxic effects of hypervitaminosis A.[195]

The effect of the dietary protein level on the embryotoxicity of orally administered vitamin A acid was examined in rats.[196] A linear relation existed between the protein level and the number of resorptions. Moreover, the number of malformations was highest with low protein levels, and the incidence of some of them correlated with the dietary protein.

Various other agents and procedures were used in these types of experiments. Hypothermia and hyperthermia,[197 198] stress due to immobilization,[199] trypan blue,[176] and urethran[200] had an enhancing effect. Somatotropic hormone, parathyroidectomy, thymectomy,[187] chondroitin sulfate,[201] and estradiol[185] showed no modifying action.

When the incidence of cleft palate was correlated with the uterine position of the fetuses, it appeared that the specimens at the vaginal end were more liable to have the malformation than those at the ovarial end.[202]

INFLUENCE OF CHEMICAL FORM, SOLVENT, AND ROUTE OF ADMINISTRATION

In experiments, vitamin A was used as vitamin A alcohol, vitamin A palmitate, vitamin A acetate, vitamin A acid, or the sodium salt derived from this. As solvents, vegetable oils, ethanol, water, or Tween 80® were used. Administration took place orally, i.p., s.c., i.m., i.v., or intra-amniotically. All the tested chemical forms were teratogenic when appropriate doses were used. The route of administration and the solvent appeard to matter insofar that preparations diluted in oil were not embryotoxic when administered i.p.[203] Comparative investigations demonstrated that vitamin A acid had the same degree of embryotoxicity as a dose of vitamin A acetate 40 times as large, if the doses were expressed in IU.[63] Because of the short half-life and the strong embryotoxicity, vitamin A acid is considered important for research into the pathophysiology of vitamin A embryotoxicity.[63 132] Abramovici pointed out that in chick embryos the embryotoxicity of the fat-soluble preparation was greater than that of a water-soluble one.[204] The embryolethal effect of the water-soluble form was increased by adding Ca or Mg salts to the solvent.

PATHOPHYSIOLOGY OF VITAMIN A EMBRYOTOXICITY

An agent can have an embryotoxic effect by affecting the maternal organism, the placenta, or the embryo itself. Therefore, several experiments were done to determine the distribution of vitamin A in female and embryo.

After adminstration of 60,000 IU on D. 12 to 14, a twofold increased vitamin A level in D. 16 fetuses was found.[205 206] A 10- to 20-fold increase was measured after administration of 72,000 IU on different days between D. 9 and 15.[207] After oral administration of 25,000 and 2500 IU to rats and mice on D. 12 or 16, the maternal serum level reached a peak after 5 hr and was back to normal after about 24 hr.[208] In this period, the fetal vitamin A content showed a 6- to 12-fold increase, when the maternal serum concentration rose beyond 250 IU/100 mℓ in the rat or beyond 210 IU/100 mℓ in the mouse.

The distribution of the vitamin in D. 16 to 18 mouse fetuses and placentae was

studied by means of autoradiography and liquid scintillation-counting after injection of ^{3}H-vitamin A acetate on D. 12.[209,210] Radioactivity was present in the retina, but not in the palatal shelves of cleft palate fetuses. However, the placentae of these fetuses contained much more radioactivity than the placentae of treated fetuses without cleft palate. Hence, it was concluded that excess vitamin A is teratogenic due to a deleterious effect on the placenta. Some other investigators performed liquid scintillation countings after oral administration of ^{3}H-vitamin A acid to pregnant mice on D. 12.[211,212] In 1 hr after administration of a nonteratogenic dose, radioactivity could be demonstrated in the embryo, the visceral yolk sac, and the chorioallantoic placenta, reaching a peak value at 2 hr in the placenta and 6 hr in the visceral yolk sac and the embryo. With administration of the teratogenic dose, the highest value in the embryo was measured after 12 hr and in the placenta and visceral yolk sac after 6 hr. For both used doses, the maximum accumulation in the embryo was two to three times as high as in the visceral yolk sac and the placenta. The teratogenic dose produced a value in the embryo which was 26-fold the value measured after administration of a nonteratogenic dose. The conclusion was drawn that a marked increase of vitamin A acid or its metabolites occurs in the embryo after administration of a teratogenic dose. Furthermore, vitamin A acid was considered to be the compound causing the teratogenic effects.

The above-mentioned experiments were done in stages, in which the choriollantoic placenta had already developed. However, in earlier stages, vitamin A was also localized 24 to 72 hr after administration of excess vitamin A in rats.[213] The characteristic autofluorescence of vitamin A was demonstrated in the yolk sac placenta and several other structures, but not in the embryo. Hence, it was concluded that in an early stage, vitamin A is teratogenic by affecting the yolk-sac placenta.

Schultz examined the yolk-sac placenta, embryo, and chorioallantoic placenta in the rat on D. 14 after administration of 60,000 IU vitamin A on D. 10 to 12.[214] No abnormalities in the lysosomal stability or in the lysosomal enzyme pattern could be demonstrated in these tissues. However, excessive tissue degeneration was observed with the electron microscope in the interdigital areas of physiological cell death.[215,216] This exaggeration of the normal process was thought to be caused by a vitamin A-induced release of the lysosomal enzymes. With electron microscopy, lesions were observed in decidua, yolk-sac epithelium, ectoplacental cone, and embryo.[217] Protrusions of the cell membrane, swollen intracellular membranes, condensed mitochondria, degenerating cells, debris, and increased intercellular space could be seen after administration of excess vitamin A. Lipid droplets were also present, and it was suggested that they represented the vitamin A accumulations found by means of fluorescence microscopy.[213]

Examination of rat embryos cultured from D. 9 for 24 to 48 hr in media with 0.5 μg/mℓ vitamin A alcohol showed that the pharyngeal arches were underdeveloped, and the rostral end of the neural tube was not closed. The closed part had multiple flexures without the formation of distinct somites,[218] Vitamin A acid added to the culture medium (0.1 μg/mℓ) produced the same type of abnormalities, but in a more severe degree.[219] The cultured treated embryos showed almost the same ultrastructural features as the material from the in vivo experiments. From this, the conclusion was drawn that excess vitamin A acts directly in the early embryo. A direct effect could also be demonstrated in cultures of older embryonic structures in which malformations of the palate[119,131,220] or of the limbs were induced by adding vitamin A to the medium.[132,159,221-223] In experiments in which the maternal and placental factors were eliminated by intra-amniotic injection on D. 14, different malformations were induced.[224] In in vitro experiments, the amniotic fluid of vitamin A-treated female rats prevented fusion of explanted palatal shelves.[225]

Impairment of the motility of cultured mesenchymal cells of the limb bud in the presence of vitamin A was proposed as a mechanism in the genesis of limb deformities.[226] Reduced cell movement in a vitamin A-containing medium was also observed in cells explanted from the primitive streak of D. 10 rat embryos.[227]

Autoradiography with ^{3}H-thymidine in rat fetuses of D. 14 after maternal treatment with 60,000 IU on D. 11 to 13 showed a reduced DNA synthesis in liver, trigeminal nucleus, palatal shelves, and cranial mesenchyme.[124] In a cell cycle study, Langman and Welch found prolongation of the mitotic and DNA-synthetic phase of neuroblasts in the fetal cerebral cortex.[162] Other autoradiographic investigations showed that in rat fetuses after administration of 6000 or 12,000 IU vitamin A on D. 14, no change in mitotic index or thymidine index occurred in telencephalon and mesencephalon.[228] However, after administration of 60,000 IU, the number of labeled cells was reduced after 2 to 15 hr, and the number of mitoses was reduced after 2 to 24 hr. An increase in degenerating cells was also observed, and at late stages, internal hydrocephalus occurred in 10% of the fetuses.

The incorporation of ^{35}S in the fetal cartilage was studied in several experiments.[116 119 125 126 229] From these papers, the conclusion can be drawn that maternal hypervitaminosis A increases the incorporation of ^{35}S, especially when heterotopic cartilage is formed. In cultured limb buds, the uptake of ^{35}S was decreased in the presence of vitamin A.[159]

Takekoshi supposed a relation between the reduction of maternal PBI in hypervitaminosis A and its teratogenic effect. From an increase in adrenal weight of treated females, it was concluded that a relation could exist between adrenal hypertrophy and the induction of congenital defects by hypervitaminosis A.[190]

HYPERVITAMINOSIS A AND HUMAN PREGNANCY

Little is known about the effects of hypervitaminosis A during human pregnancy. Bernhardt and Dorsey described a newborn whose mother had taken 50,000 IU/day in the 4th to 9th month of pregnancy.[230] The child had ureter duplication on the left, accompanied by bilateral hydroureter and hydronephrosis. Some other papers described the baby of a woman who had daily taken 40,000 IU vitamin A from the sixth to the tenth week of pregnancy.[231 232] The child had hypoplastic orifices of the ureter, an apical diverticle of the urinary bladder, and hydroureter with hydronephrosis. Malformations of heart and palate were mentioned by Morriss and Thomson.[233] Multiple craniofacial malformations occurred in a baby whose mother ingested a very high dose of vitamin A in the second month of gravidity. After ingestion, clinical symptoms of vitamin A intoxication (headache, desquamation of the skin, and subfrebrile temperature) had developed.[234]

Gal et al. examined serum samples drawn 1 week post partum from women who had delivered a child with a spina bifida.[235] The vitamin A level was raised significantly in these cases. Furthermore, in the liver of fetuses with a nervous system malformation, an increased vitamin A concentration was also measured. On account of these data, it was concluded that vitamin A can have an embryotoxic effect in humans. From experiments, it appeared that in vitro a very low concentration of vitamin A alcohol (0.5 μg/ml), close to the standard value in the human serum (0.3 to 0.5 μg/ml), has a teratogenic effect.[218] Furthermore, it was stressed that some maternal factors (increased intake, diminished liver storage capacity, and reduced production of retinol-binding protein) can elevate the level of vitamin A in maternal serum and fetus.[233]

It can be concluded that at this moment no definitive proof to the teratogenic effect of excess vitamin A in man exists. However, considering the results of experiments

with different species of animals and the scarce data in man, hypervitaminosis A in pregnancy may have serious consequences for the developing embryo and fetus.

REFERENCES

1 **Kalter, H and Warkany, J.**, Experimental production of congenital malformations in mammals by metabolic procedure, *Physiol Rev* , 39, 69, 1959
2 **Cahen, R L.**, Evaluation of the teratogenicity of drugs, *Clin Pharmacol Ther* , 5, 480, 1964
3 **Kalter, H** , *Teratology of the Central Nervous System*, University of Chicago Press, Chicago, 1968, chap 3
4 **Warkany, J.**, *Congenital Malformations Notes and Comments*, Year Book Medical Publishing Chicago, 1971
5 **Moore, T** , *Vitamin A*, Elsevier, Amsterdam, 1957
6 **Dingle, J. T and Lucy, J A.**, Vitamin A, carotenoids and cell function, *Biol Rev* , 40, 422, 1965
7 **DeLuca, H F. and Zile, M** , Aspects of teratology of vitamin A acid (β all trans retinoic acid), *Acta Derm Venereol Suppl* , 74, 13, 1975
8 **Giroud, A.**, Vitamine A et tératogénèse Teratogenesis Symposion, *Bull Schweiz Akad Med Wiss* 20 440, 1964
9 **Langman, J and Welch, G. W** , Effect of vitamin A on development of the central nervous system *J Comp Neurol* , 128, 1 1966
10 **Cohlan, S Q.**, Excessive intake of vitamin A during pregnancy as a cause of congenital anomalies in the rat, *Amer J Dis Child* , 86, 348, 1953
11 **Cohlan, S. Q.**, Excessive intake of vitamin A as a cause of congenital anomalies in the rat, *Science*, 117, 535, 1953
12 **Cohlan, S. Q.**, Congenital anomalies in the rat produced by excessive intake of vitamin A during pregnancy, *Pediatrics*, 13, 556, 1954
13 **Giroud, A. and Martinet, M.**, Fentes du palais chez l'embryon de rat par hypervitaminose A, *C R Soc Biol* , 148, 1742, 1954
14 **Giroud, A. and Martinet, M.**, Hypervitaminose A et anomalies chez le foetus de rat, *Int Z Vitaminforsch*, 26, 10, 1955
15 **Giroud, A. and Martinet, M.**, Malformations diverses du foetus de rat suivant les stades d'administration de vitamine A en excès, *C R Soc Biol* , 149, 1088, 1955
16 **Giroud, A. and Martinet, M.**, Influence tératogène de l'hypervitaminose A selon les stades du développement embryonnaire, *C R Assoc Anat* 42 562, 1955
17 **Giroud, A and Martinet, M** , Tératogénèse par hautes doses de vitamine A en fonction des stades du développement, *Arch Anat Microsc Morphol Exp* , 45, 77, 1956
18 **Giroud, A and Martinet, M.**, Extension à plusieurs especes de mammifères des malformations embryonnaires par hypervitaminose A, *C R Soc Biol* , 153, 201, 1959
19 **Giroud, A and Martinet, M** , Tératogénèse par hypervitaminose A chez le rat, la souris, le cobaye et le lapin, *Arch Fr Pediatr* , 16, 971, 1959
20 **Giroud, A. and Martinet, M.**, Anencéphalie chez la souris par hypervitaminose A, *C R Assoc Anat* 6, 288, 1960
21 **Giroud, A and Martinet, M.**, Anencéphalie expérimentale chez la souris et comparaisons avec l'anencephalie chez l'homme, *Acta Anat* , 43, 358, 1960
22 **Giroud, A. and Martinet, M** , Action tératogene de l'hypervitaminose A chez la souris en fonction du stade embryonnaire, *C R Soc Biol* , 154, 1353, 1960
23 **Murakami, U. and Kameyama, Y.**, Malformations of the mouse fetus caused by hypervitaminosis A of the mother during pregnancy, *Arch Environ Health*, 10, 732, 1965
24 **Shenefelt, R E.**, Morphogenesis of malformations in hamsters caused by retinoic acid relation to dose and stage at treatment, *Teratology*, 5, 103, 1972
25 **Marin-Padilla, M. and Ferm, V. H.**, Somite necrosis and developmental malformations induced by vitamin A in the golden hamster, *J Embryol Exp Morphol* , 13, 1, 1965
26 **Giroud, A and Martinet, M** , Répercussions de l'hypervitaminose A chez l'embryon de lapin, *C R Soc Biol* , 152, 931, 1958
27 **Auroux, M., Alnot, M. O., and Roussel, C.**, Etude des commissures télencéphaliques chez le foetus de rat anencéphale, *Arch Anat* , 48, 221, 1965

28 **Baba, T and Araki, E,.** Morphogenesis of malformation due to excessive vitamin A I Morphogenesis of exencephaly, *Osaka City Med J* , 5, 9, 1959

29 **Giroud, A.,** Malformazioni cefaliche da eccesso di vitamina A e considerazioni morfologiche e patogenetiche, *Minerva Chir* , 13, 927, 1958

30 **Giroud, A.,** Causes and morphogenesis of anencephaly, in *CIBA Found Symp Congenital Malformations*, Wolstenholme, G E W and O'Connor, C M , Eds , Little, Brown, Boston, 1960

31 **Giroud, A., Delmas, A , and Martinet, M.,** Etude morphogénètique sur des embryons anencéphales, *Arch Anat* , 42, 203, 1959

32 **Giroud, A and Martinet, M.,** Malformations embryonnaires par hypervitaminose A, *Arch Fr Pédiatr* , 12, 292, 1955

33 **Giroud, A. and Martinet, M.,** Hypovitaminose et hypervitaminose A chez le jeune et chez L'embryon, *Etud Neo Natal* , 5, 54, 1956

34 **Giroud, A. and Martinet, M.,** Morphogénèse de l'anencéphalie, *Arch Anat Microsc Morphol Exp* , 46, 246, 1957

35 **Giroud, A. and Martinet, M.,** Stades évolutifs de l'ébauche encéphalique dans les cas de l'anencéphalie, *C R Soc Biol* , 151, 872, 1957

36 **Giroud, A. and Martinet, M.,** Stades de développment de l'anencéphalie, *C R Assoc Anat* , 44, 301, 1958

37 **Rebello, M. A ,** Rôle des pressions liquidiennes dans la morphogénèse de l'encéphale, *C R Soc Biol* , 152, 312, 1958

38 **Giroud, A. and Martinet, M.,** Anencéphalie chez la souris par hypervitaminose A et considérations générales, *Rev Neurol* , 103, 356, 1960

39 **Ferm, V. H.,** The rapid detection of teratogenic activity, *Lab Invest* , 14, 1500, 1965

40 **Morriss, G. M.,** Morphogenesis of the malformations induced in rat embryos by maternal hypervitaminosis A, *J Anat* , 113, 241, 1972

41 **Morriss, G. M.,** The ultrastructural effects of excess maternal vitamin A on the primitive streak stage rat embryo, *J Embryol Exp Morphol* , 30, 219, 1973

42 **Geelen, J. A G.,** Vitamin A-induced anomalies in young rat embryos, *Acta Morphol Neerl Scand* , 11, 233, 1973

43 **Theodosis, D and Fraser, F. C.,** Pathogenesis of vitamin A-induced exencephaly in the mouse, *Teratology*, 7, 29a, 1973

44 **Marin-Padilla, M.,** Mesodermal alterations induced by hypervitaminosis A, *Embryol Exp Morphol* , 15, 261, 1966

45 **Geelen, J. A. G ,** Skullbase malformations in rat fetuses with hypervitaminosis A-induced exencephaly, *Teratology*, 7, 49, 1973

46 **Geelen, J. A. G.,** The skullbase in vitamin A-induced exencephaly, *Acta Univ. Carol Med Monogr* , 56, 157, 1973

47 **Geelen, J. A G.,** Malformations of the skullbase in exencephalic rat fetuses, *Teratology*, 8, 221, 1973

48 **Giroud, A. and Martinet, M.,** Production d'un excès de liquide amniotique dans l'anencephalie, *C R Soc Biol* , 149, 452, 1955

49 **Giroud, A. and Martinet, M.,** Hydramnios et anencéphalie, *Gynecol and Obstet.*, 54, 391, 1955

50 **Singh, S. and Singh, G,.** Lethality and teratogenicity of hypervitaminosis A in rats, *J Anat , Soc India*, 23, 89, 1974

51 **Gool, J. van, Peters, P. W. J., and Geelen, J. A. G.,** α_MFP in amniotic fluid of rats with hypervitaminosis A induced neural tube defects, in *Protides of the Biological Fluids*, Peeters H , Ed , Pergamon Press, Oxford, 1976

52 **Peters, P. W. J. and Geelen, J. A. G.,** α and α_m fetoprotein levels in amniotic fluid of vitamin-A induced anencephalic rat fetuses and in maternal serum, *Teratology*, 12, 332, 1975

53 **Peters, P. W. J. and Geelen, J. A. G.,** Morphological aspects of late stages of vitamin A induced anencephaly, *Teratology*, 14, 379, 1976

54 **Giroud, A., Martinet, M., and Lefebvres-Boisselot, J.,** Relations entre les anomalies de fermeture du tube nerveux et les deficiences de la voute osseuse du nevraxe et l'indifferenciation de l'epiderm susjacent, *Arch. Anat , Histol Embryol* , 43, 202, 1960.

55 **Giroud, A., Martinet, M., and Lefebvres-Boisselot, J.,** Différenciation de la peau, de la vôute du crâne et du canal vertébral dans les anomalies du névraxe, *C R Soc Biol* , 154, 939, 1960

56 **Giroud, A., Martinet, M., and Solère, M.,** Encéphalocèles, méningocèles par hypervitaminose A et considérations cliniques, *Rev Neurol* , 98, 181, 1958

57 **Giroud, A., Martinet, M., and Solère, M.,** Anencéphalie, encéphalocèles, méningocèles par hypervitaminose A, *Arch Fr Pédiatr* , 15, 835, 1958

58 Giroud, A. and Roux, C., Différenciation de l'épiderme et aplasie cutanée circonscrite, *Bull Soc Fr Dermatol Syphiligr*, 68, 197, 1961

59 McLaurin, R. L., Parietal cephalocoeles, *Neurology*, 144, 764, 1964

60 Benito-Arranz, S., El organo pericomisural en la hidrocefalia experimental, *Arch Esp Morfol*, 18, 113, 1963

61 Benito-Arranz, S., Hidrocefalia experimental por hipervitaminosis A, *Bol Patol Med*, 4, 70, 1964

62 Kalter, H., Congenital malformations of the central nervous system, *Am J Clin Nutr*, 12, 264, 1963

63 Kochhar, D. M., Teratogenic activity of retinoic acid, *Acta Pathol Microbiol Scand*, 70, 398, 1967

64 Giroud, A., Delmas, A., and Martinet, M., Cyclocéphalie morphogénèse et mécanisme de sa production, *Arch Anat Histol Embryol*, 46, 295, 1963

65 Giroud, A. and Martinet, M., Au sujet d'embryons cyclocéphales, *C R Soc Biol*, 155, 2074, 1961

66 Giroud, A. and Martinet, M., Nerfs olfactifs et bulbe olfactif, *Arch Anat Histol Embryol*, 45, 193, 1962.

67 Giroud, A., Martinet, M., and Deluchat, C., Mécanisme de développement de bulbe olfactif, *Arch Anat, Histol Embryol*, 48, 205, 1965

68 Giroud, A. and Roux, C., Système chordal et développement de l'hypophyse, *Arch Anat Microsc Morphol Exp*, 48, 101, 1959

69 Giroud, A. and Roux, C., Système chordal et morphogénèse de la tête chez l'embryon normal et chez les monstres doubles, *Arch Anat Histol Embryol*, 42, 285, 1959

70 Eguchi, Y., Morikawa, Y., and Hashimoto, Y., Lack of hypertrophy of the adrenal in exencephalic fetal rats subjected to hypervitaminosis A and then to maternal adrenalectomy, *Endocrinology*, 88, 1526, 1971.

71 Eguchi, Y., Suzuki, S., Morikawa, Y., and Hashimoto, Y., Experimental formation of goiter in exencephalic fetal rats subjected to maternal hypervitaminosis A, *Endocrinology*, 88, 261, 1971

72 Fujita, T., Eguchi, Y., Morikawa, Y., and Hashimoto, Y., Hypothalamic hypophyseal adrenal and thyroid systems observations in fetal rats subjected to hypothalamic destruction, brain compression and hypervitaminosis A, *Anat Rec*, 166, 659, 1970

73 Eguchi, Y., Hirai, O., Morikawa, Y., and Hashimoto, Y., Critical time in the hypothalamic control of the pituitary-adrenal system in fetal rats observations in fetuses subjected to hypervitaminosis A and hypothalamic destruction, *Endocrinology*, 93, 1, 1973

74 Giroud, A., Martinet, M., and Deluchat, C., Fissuration faciale médiane, *Arch Anat Histol Embryol*, 52, 207, 1969

75 Kalter, H and Deuschle, F. M., Congenital malformations experimentally induced by hypervitaminosis A, in *Environmental Variables in Oral Disease, No 81*, Kreshover J and McClure, F Eds, American Association for the Advancement of Science, Washington, D C, 1966

76 Robens, J. F., Teratogenic effects in hypervitaminosis A in the hamster and the guinea pig, *Toxicol Appl Pharmacol*, 14, 640, 1969

77 Robens, J. F., Teratogenic effects in hypervitaminosis A in the hamster and the guinea pig, *Toxicol Appl Pharmacol*, 16, 88, 1970

78 Baba, T. and Kotani, S., Morphogenesis of malformation due to excessive vitamin A III Morphogenesis of an-or microphthalmos, *Osaka City Med J*, 5, 135, 1959

79 Kotani, S., Morphogenesis of ophtalmic malformation of rats produced by A-hypervitaminosis, *J Osaka City Med Center*, 7, 132, 1958

80 Miki, T., Morphogenetic study of congenital ocular malformations in rats induced by excessive vitamin A, with special references to exophthalmos, *Osaka City Med J*, 14, 1, 1968

81 Giroud, A. and Martinet, M., Malformations oculaires avec fibrose du vitré chez des embryons de lapin soumis à l'hypervitaminose A, *Bull Soc Ophtalmol Paris*, 3, 191, 1959

82. Giroud, A., Martinet, M., and Roux, C, Migrations anormales des fibres optiques et considérations générales, *Arch Anat Histol Embryol*, 45, 177, 1962

83 Giroud, A., Martinet, M, and Roux, C., Considérations sur les anomalies des nerfs optiques et olfactifs, *C R Assoc Anat* 48 661 1962

84 Giroud, A., Martinet, M., and Deluchat, C., Anomalies de nerf optique, *Bull Soc Ophtalmol Paris*, 66, 1, 1966

85 Kalter, H., Teratogenically induced congenital aphakia, *Exp Eye Res*, 3, 228, 1964

86 Fantel, A. G., Shephard, T H, Newell-Morris, L. L., and Moffet, B. C., Teratogenic effects of retinoic acid in monkeys, *Pediatr Res*, 9, 358, 1975

87 Fantel, A. G, Shephard, T. H., Newell-Morris, L. L, and Moffet, B. C., Teratogenic effects of retinoic acid in pigtail monkeys *(Macaca nemestrina)* I General features, *Teratology* 15 65 1977

88 Palludan, B., Swine in teratological studies in *Swine in Biomedical Research* Bustad L K and McClellan R O, Eds, Frayn Printing Seattle, 1966

89 **Baba, T and Kuzukawa, S**, Morphogenesis of malformation due to excessive vitamin A V Embryological studies on the inner ear malformation of rats due to excessive vitamin A, *Osaka City Med J*, 8, 105, 1962

90 **Baba, T and Nakamura, S.**, Changes in the rat fetus inner ear due to excessive vitamin A, *Osaka City Med J*, 5, 1, 1959

91 **Kuzukawa, S** Embryological studies on the inner ear malformation of rats due to excessive vitamin A *J Osaka City Med Center* 9 1469 1960

92 **Nakamura, S.**, Experimental studies on auditory organs, especially on inner ear of rat fetuses born to mother animals fed with excessive vitamin A, *J Osaka City Med Center*, 7, 486, 1958

93 **Giroud, A. and Martinet, M.**, Malformations de la face et hypervitaminose A, *Rev Stomatol Chir Maxillo Fac*, 57, 454, 1956

94 **Hayashi, Y**, Malformations of the ear in mouse fetus caused by maternal hypervitaminosis A *Cong Anom* 12 73 1972

95 **Kalter, H**, Teratogenic action of hypervitaminosis A in strains of inbred mice, *Anat Rec*, 133, 396, 1959

96 **Kalter, H and Warkany, J**, Experimental production of congenital malformations in strains of inbred mice by maternal treatment with hypervitaminosis A, *Am J Pathol*, 38, 1, 1961

97 **Kalter, H.**, Hypervitaminosis A-induced internal congenital malformations in strains of inbred mice, *Anat Rec*, 134, 589, 1959

98 **Kalter, H.**, The teratogenic effects of hypervitaminosis A upon the face and mouth of inbred mice, *Ann N Y Acad Sci*, 85, 42, 1960

99 **Kameyama, Y**, Malformations of facial bones in the mouse caused by hypervitaminosis A of the pregnant mother *Cong Anom* 6, 110 1966

100 **Deuschle, F M., Geiger, J F, and Warkany, J**, Analysis of an anomalous oculodentofacial pattern in newborn rats produced by maternal hypervitaminosis A, *J Dent Res*, 38, 149, 1959

101 **Ihara, T., Mizutani, M., Tanaka, S., and Kaziwara, K**, Effects of hypervitaminosis A on several species of experimental animals with special reference to the oculofacial syndrome, *Cong Anom*, 6, 91, 1966

102 **Abramovich, A.**, Changes in facial structures of rats caused by supplements of vitamin A and *Lathyrus Odoratus*, *J Dent Res*, 52, 300, 1973

103 **Knudsen, P A.**, Congenital malformations of the jaws and related structures in exencephalic mouse embryos with anomalous molar germs, induced by hypervitaminosis A, *Acta Odontol Scand*, 24, 677, 1966

104 **Wilson, J. G.**, Use of rhesus monkeys in teratological studies, *Fed Proc Fed Am Soc Biol*, 30, 104, 1971

105 **Knudsen, P.A.**, Congenital malformations of upper incisors in exencephalic mouse embryos induced by hypervitaminosis A I Types and frequency, *Acta Odontol Scand*, 23, 71, 1965

106 **Knudsen, P A.**, Congenital malformations of upper incisors in exencephalic mouse embryos, induced by hypervitaminosis A II Morphology of fused upper incisors, *Acta Odontol Scand*, 23, 391, 1965

107 **Knudsen, P. A.**, Fusion of upper incisors at bud or cap stage in exencephalic mouse embryos induced by hypervitaminosis A, *Acta Odontol Scand*, 23, 549, 1965

108 **Knudsen, P. A.**, Congenital malformations of lower incisors and molars in exencephalic mouse embryos, induced by hypervitaminosis A, *Acta Odontol Scand*, 24, 55, 1966

109 **Knudson, P. A.**, The infraseptal nasal glands in mouse embryos with dental malformations and exencephaly induced by hypervitaminosis A, *Acta Odontol Scand*, 25, 247, 1967

110 **Knudsen, P. A.**, Dental malformations in rat embryos with exencephaly induced by hypervitaminosis A, *Acta Odontol Scand*, 25, 267, 1967

111 **Knudsen, P. A.**, Tooth germs in non-exencephalic siblings of mouse embryos with exencephaly induced by hypervitaminosis A, *Acta Odontol Scand*, 25, 659, 1967

112 **Knudson, P. A**, Dental anomalies in mouse embryos with hydrocephalus induced by hypervitaminosis A, *Acta Odontol Scand*, 25, 677, 1967

113 **Fujii, T.**, Effects of vitamin A on the dental morphogenesis in the mouse fetus, *Cong Anom*, 14, 293, 1974

114 **Fujii, T.**, Effects of vitamin A on the dental system in mice, *Teratology*, 10, 81, 1974

115 **Masi, P L.**, Anomalie dentali nella otocefalia e nella ciclocefalia sperimentali del topo e del coniglio *Atti Accad Fisiocrit, Siena Sez Med Fis*, 14, 403, 1965

116 **Kochhar, D. M. and Johnson, E. M.**, Morhological and autoradiographic studies of cleft palate induced in rat embryos by maternal hypervitaminosis A, *J Embryol Exp Morphol*, 14, 223, 1965

117 **Kochhar, D. M**, Effects of Maternal Hypervitaminosis A on Cleft Palate Formation in Rat Embryos, Ph D thesis, University of Florida, Gainesville, *Diss Abstr*, 25, 5490, 1964

118 **Walker, B. E. and Crain, B.**, Effects of hypervitaminosis A on palate development in two strains of mice, *Am J Anat*, 107, 49, 1960

119 **Nanda, R.**, The normal palate and induced cleft palate in rat embryos, Ph D thesis, University of Nijmegen, The Netherlands, 1969

120 **Lotosh, E. A**, Cleft palate caused in embryos by administering large doses of vitamin A combined with cortisone to pregnant mice, *Stomatologiya (Moscow)*, 44, 30, 1965

121 **Lotosh, E. A**, Effect of maternal hypervitaminosis A on the results of injury to the palatal Anlage in mammalian embryos, *Dokl Biol Sci*, 169, 476, 1966

122 **Lotosh, E. A**, Hypervitaminosis A in pregnant mice of line C3HA and developmental anomalies of the palate and jaws, *Bull Exp Biol Med (USSR)*, 62, 1297, 1967

123 **Lotosh, E. A.**, Development of palate in normal murine embryo and influence of various agents on the conditions of palatine plates at different stages of embryonic development, *Acta Chir Plast*, 10, 1, 1968

124 **Kochhar, D. M.**, Studies of vitamin A-induced teratogenesis effects on embryonic mesenchyme and epithelium, and on incorporation of ^{3}H thymidine, *Teratology*, 1, 299, 1968

125 **Kochhar, D M., Larsson, K. S., and Bostrom, H**, Embryonic uptake of ^{35}S sulfate change in level following treatment with some teratogenic agents, *Biol Neonat*, 12, 41, 1968

126 **Nanda, R.**, The role of sulfated mucopolysaccharides in cleft palate production, *Teratology*, 3, 237, 1970

127 **Nanda, R., van der Linden, F. P G. M., and Jansen, H. W. B**, Production of cleft palate with dexamethason and hypervitaminnosis A in rat embryos, *Experientia*, 26, 1111, 1970

128 **Ross, L. M. and Walker, B E.**, Movement of palatine shelves in untreated and teratogen-treated mouse embryos, *Am J Anat*, 121, 509, 1967

129 **Shih, L. Y., Trasler, D G., and Fraser, F C.**, Mandible growth during palate closure in normal and experimentally induced cleft palate in A/J and C57BL mice, *Teratology*, 4, 241, 1971

130 **Shih, L Y., Trasler, D G, and Fraser, F. C.**, Relation of mandible growth to palatal closure in mice, *Teratology*, 9, 191, 1974

131 **Nanda, R.**, Effect of vitamin A on the potentiality of rat palatal processes to fuse in vivo and in vitro, *Cleft Palate J*, 4, 123, 1974

132 **Kochhar, D M.**, Limb development in mouse embryos I Analysis of the teratogenic effects of retinoic acid, *Teratology*, 7, 289, 1973

133 **Diewert, V. M.**, Palatine arteries in normal and cleft palate rat fetuses, *J Dent Res*, 52, 278, 1973

134 **Diewert, V. M.**, Effects of three teratogens on development of arteries in the secondary palate in rats, *Teratology*, 13, 113, 1976

135 **Abramovich, A. and Devoto, F. C. H.**, Comparación entre fisuras palatinas producidas por hipervitaminosis A y latirismo, *Rev Soc Argent Biol*, 43, 94, 1967

136 **Abramovich, A. and Devoto, F. C H.**, Ectopic maxillofacial chondrogenesis in fetuses of rats treated with high doses of vitamin A, *Rev Asoc Odont Argent*, 55, 237, 1967

137 **Abramovich, A. and Devoto, F. C. H.**, Lésions congénitales maxillo-faciales obtenues avec différentes doses de vitamine A, *C R Soc Biol*, 162, 2011, 1968

138 **Baba, T. and Yukioka, K.**, Effect of embryonal environment on development XI Cleft palate due to A-hypervitaminosis, *Trans Soc Pathol Jpn*, 47, 552, 1958

139 **Baba, T. and Yukioka, K.**, Morphogenesis of malformation due to excessive vitamin A II Morphogenesis of cleft palate and its critical phase, *Osaka City Med J*, 5, 91, 1959

140 **Filippi, B.**, Ipervitaminosi A e palatoschisi, *Minerva Chir*, 11, 1314, 1956

141 **Koval, A. V.**, Development of the palate in rats under normal conditions and with an experimental congenital cleft palate, *Stomatologiva (Moscow)*, 45, 34, 1966

142 **Millen, J. W. and Woollam, D. H. M**, Potency of parenteral vitamin A, *Nature (London)*, 185, 249, 1960

143 **Lorente, C. E. and Miller, S. A.**, Effects of hypervitaminosis A on rat palatal development, *Fed Proc Fed Am Soc Exp Biol*, 35, 421, 1976

144 **Poswillo, D. and Roy, L. J.**, The pathogenesis of cleft palate An animal study, *Br J Surg*, 52, 902, 1965

145 **Pourtois, M.**, La fusion des êretes palatines et son alteration par quelques agents tératogènes, *Arch Biol*, 79, 105, 1968

146 **Yukioka, K.**, Morphogenesis of experimental cleft palate due to excessive vitamin A, *J Osaka City Med Center*, 7, 725, 1958

147 **Giroud, A., Martinet, M., and Roux, C.**, Malformations urinaires dans l'hypervitaminose A, *C R Soc Biol*, 151, 1811, 1957

148. **Giroud, A., Martinet, M., and Roux, C.**, Urêtéro-hydronéphrose expérimentale chez l'embryon par hypervitaminose A, *Arch Fr Pédiatr*, 15, 540, 1958

149 **Giroud, A., Martinet, M, and Roux, C**, Considérations à propose des hydronéphroses par hypervitaminose A chez l'embryon de rat *Arch Anat Pathol*, 7, 113 1959

150 **Baba, T and Tsuruhara, T** , Morphogenesis of malformation due to excessive vitamin A IV Morphogenesis of congenital hydronephrosis and hydroureter, *Osaka City Med J* , 5, 219 1959

151 **Tsuruhara, T** , An embryological study on the abnormalities of the urinary tract system of rat due to excessive vitamin A, *J Osaka City Med Center* 8 1379 1959

152 **Kalter, H** , Congenital malformations of the rectum and urogenital system induced by maternal hypervitaminosis A in strains of inbred mice, *Anat Rec* , 136, 219 1960

153 **Love, A M and Vickers, T H** , Hypervitaminosis A dysmelia in rats *Br J Exp Pathol* 52 656 1971

154 **Mizutani, M , Ihara, T , and Kaziwara, K** , Effect of hypervitaminosis A on the fetus of several species of rodents with special reference to malformation of the extremities *Jpn J Genet* , 41 141, 1966

155 **Nakamura, H , Yamawaki, H , Fujisawa, H , and Yasuda, M** Effects of hypervitaminosis A on developing mouse limb buds ultrastructural observations, *Teratology* 8 100 1973

156 **Nakamura, H , Yamawaki, H , Fujisawa, H , and Yasuda, M** , Effects of hypervitaminosis A on developing mouse limb buds ultrastructural observations *Cong Anom* 13 181 1973

157 **Nakamura, H , Yamawaki, H , Fujisawa, H , and Yasuda, M** , Effects of maternal hypervitaminosis A upon developing mouse limb buds II Electron microscopic investigation *Cong Anom* 14 271 1974

158 **Yamawaki, H , Nakamura, H , Fujisawa, H , and Yasuda, M** , Effects of maternal hypervitaminosis A upon developing mouse limb buds I Light microscopic investigation *Cong Anom* 14 13 1974

159 **Nakamura, H** , Analysis of limb anomalies induced in vitro by vitamin A (retinol) in mice *Teratology*, 12, 61, 1975

160 **Terashima, Y , Hirose, J , and Nogami, H** , Dyschondrosteosis produced in the rat fetus by excessive vitamin A, *Teratology*, 10, 100, 1974

161 **Roux, C and Martinet, M** , Syndrome de regression caudale chez l'animal, *Arch Fr Pédiatr* , 19, 781, 1962

162 **Langman, J and Welch, G W** , Excess vitamin A and development of the cerebral cortex, *J Comp Neurol* , 131, 15, 1967

163 **Malakhovskii, V G** , Behavioral disturbances in rats receiving teratogenic agents antenatally, *Bull Exp Biol Med* , 68, 1230, 1969

164 **Malakhovskii, V G** , Antenatal effect of pyrimethamine and vitamin A on behavior of rat progeny *Bull Exp Biol Med* , 71, 254, 1971

165 **Prozorovskii, V B and Malakhovskii, V G** , The range of teratogenic action as an index of the hazard of using a medicinal substance during pregnancy, *Akush Ginekol* , 46, 52, 1970

166 **Butcher, R E , Brumer, R L , Roth, T , and Kimmel, C A** , A learning impairment associated with maternal hypervitaminosis A in rats, *Life Sci* , 11, 141, 1972

167 **Hutchings, D E., Gibbon, J , and Kaufman, M** , Maternal vitamin A excess during the early fetal period Effects on learning and development in the offspring, *Dev Psychobiol* , 6, 445, 1973

168 **Hutchings, D E and Gaston, J** , The effects of vitamin A excess administered during the mid fetal period on learning and development in rat offspring, *Dev Psychobiol* , 7, 225 1974

169 **Vorhees, C V** , Behavioral effects of maternal hypervitaminosis A in rats, *Teratology* 10, 169, 1974

170 **Berdjis, C C** , Late effects of hypervitaminosis A in the rat Disturbance and retardation in the normal growth of offspring, *Arch Pathol* , 66, 278, 1958

171 **Kameyama, Y** , Strain differences in malformations of the extremities of the mouse fetus caused by X radiation, hypoxia or hypervitaminosis A of the mother during pregnancy *Annu Rep Res Inst Environ Med Nagoya Univ* , 13, 49, 1964

172 **Kameyama, Y , Nogami, H , and Murakami, U** , Study of micromelias in the mouse fetus caused by X radiation, hypoxia, trypan blue injection or hypervitaminosis A upon mother animals during pregnancy, *Annu Rep Res Inst Environ Med Nagoya Univ* , 12, 29, 1964

173 **Nolen, G A** , Variations in teratogenic response to hypervitaminosis A in three strains of the albino rat, *Food Cosmet Toxicol* , 7, 209, 1969

174 **Ohmori, K** , Changes in incidence of the manifestation of anatomical variations of the vertebrae in the mouse fetus caused by X radiation or maternal hypervitaminosis A, *Cong Anom* 7 219 1967

175 **Giroud, A and Martinet, M** , Legerete de la dose teratogene de la vitamine A *C R Soc Biol* , 156, 449, 1962

176 **Wilson, J G** , Teratogenic interaction of chemical agents in the rat, *J Pharmacol Exp Ther* 144, 429, 1964

177 **Committee on Animal Nutrition, Nutrient requirements of domestic animals Nutrient requirements of laboratory animals, National Academy of Sciences — National Research Council, Washington, D C , 1962**

178 Committee on Animal Nutrition, Nutrient requirements of domestic animals Nutrient requirements of rabbits, National Academy of Sciences — National Research Council, Washington, D C 1966
179 Committee on Animal Nutrition, Nutrient requirements of domestic animals Nutrient requirements of dogs, National Academy of Sciences — National Research Council, Washington, D C , 1962
180 Food and Nutrition Board, Recommended dietary allowances, National Academy of Sciences — National Research Council, Washington, D C , 1958
181 **Amano, S., Shimizu, S., Takaya, M., Sakagami, T., and Akiyama, N.**, Congenital malformation in rats due to hypervitaminosis A in combination with trypan blue and cortisone acetate, *J Osaka City Med Center* , 12, 303, 1963
182 **Millen, J. W. and Woollam, D. H. M.**, Insulin-cortisone relationship in experimental teratogenesis, *Nature (London)*, 181, 418, 1957
183 **Millen, J. W. and Woollam, D. H. M.**, Influence of cortisone on teratogenic effects of hypervitaminosis A, *Br Med J* , 2, 196, 1957
184 **Woollam, D. H. M. and Millen, J. W.**, Effect of cortisone on the incidence of cleft palate induced by experimental hypervitaminosis A, *Br Med J* , 2, 197, 1957
185 **Woollam, D. H. M. and Millen, J. W.**, The modification of the activity of certain agents exerting a deleterious effect on the development of the mammalian embryo, in *CIBA Found Symp on Congenital Malformations*, Wolstenholme, G E W and O'Connor, C M , Eds , Little, Brown, Boston, 1960
186. **Yorioka, T.**, Effects of some drugs on experimental malformations due to hypervitaminosis A, *J Osaka City Med Center*, 14, 527, 1965
187 **Cohlan, S. Q and Stone, S. M.**, Observations on the effect of experimental endocrine procedures on the teratogenic action of hypervitaminosis A in the rat, *Biol Neonat* , 3, 330, 1961
188. **Woollam, D. H. M. and Millen, J. W.**, Influence of 4-methyl-2-thiouracil on the teratogenic activity of hypervitaminosis A, *Nature (London)*, 181, 992, 1958
189 **Krazitsjka, O. P** , Teratogenic action of hypervitaminosis and hydrocortisone in conditions of experimental hypothyreosis of the maternal organism, *Farm Zh (Kiev)*, 26, 71, 1971
190 **Takekoshi, S** , The mechanism of vitamin A induced teratogenesis, *J Embryol Exp Morphol* , 12, 263, 1964
191 **Millen, J. W. and Woollam, D H. M.**, Effect of vitamin B complex on the teratogenic activity of hypervitaminosis A, *Nature (London)*, 182, 940, 1958
192 **Yamaguchi, T.**, Effects of riboflavine, pyridoxine and folic acid on the incidence of malformations in the rat fetus caused by maternal hypervitaminosis A, *Cong Anom* , 8, 175, 1968
193 **Sudo, T.**, Suppressive effect of vitamin E on experimental micromelia produced by hypervitaminosis A, *Congr Anom* , 8, 163, 1968
194 **Yamaguchi, T.**, Effects of vitamin B_{12} on the incidence of malformations in the rat fetus caused by hypervitaminosis A in the pregnant mother, *Cong Anom* , 8, 19, 1968
195 **Mitolo, M. and Mastrozilli, A.**, Lack of maternal vitamin balance and embryonal malformations. I The effects of experimental A avitaminosis or hypervitaminosis, *Riv Biol* , 60, 205, 1967
196 **Nolen, G. A.**, The effects of various levels of dietary protein on retinoic acid-induced teratogenicity in rats, *Teratology*, 5, 143, 1972
197 **Ferm, V. H.**, Potentation of the teratogenic effect of vitamin A with exposure to low environmental temperature, *Life Sci* , 6, 493, 1967
198 **Esaki, K., Tanioka, Y., and Monura, T.**, Effects of the change of the environmental temperature on the incidence of cleft palate in the mouse fetuses induced by hypervitaminosis A, *Cong Anom* , 8 13, 1968
199. **Hartel, A. and Hartel, G.**, Experimental study of teratogenic effect of emotional stress in rats, *Science*, 132, 1483, 1960
200 **Takekoshi, S.**, Effects of urethan on the teratogenic action of hypervitaminosis A, *Gunma J Med Sci* , 14, 210, 1965
201. **Ishii, H., Kamei, T., and Omae, S.**, Effects of concurrent administrations of chondroitin sulfate with cortisone, vitamin A or noise stimulation on fetal development of the mouse, *Gunma J Med. Sci.*, 11, 259, 1962
202. **Woollam, D. H. M. and Millen, J. W.**, Influence of uterine position on the response of the mouse embryo to the teratogenic effects of hypervitaminosis A, *Nature (London)*, 190, 184, 1961.
203 **Gebauer, H.**, Zur A-hypervitaminose und Schwangerschaft, *Pharmazie*, 9, 684, 1954
204 **Abramovici, A.**, Considerations pharmocologiques sur l'effet embryotoxique de l'hypervitaminose A chez le poulet, *Teratology*, 4, 482, 1971
205 **Giroud, A., Gounelle, H., and Martinet, M.**, Concentration de la vitamine A chez la mère et le foetus au cours de la teratôgénèse par hypervitaminose A, *C. R Soc Biol* , 150, 2064, 1956
206 **Giroud, A., Gounelle, H., and Martinet, M.**, Donnees quantitatives sur le taux de la vitamine A chez

le rat lors d'expériences de tératogénèse par hypervitaminose A, *Bull Soc Chim Biol.*, 39, 331, 1957

207 Opuszyńska, T. and Rusiecka, M , Effect of excess vitamin A on the occurrence of developmental defects in rats, *Rocz Zak Hig* , 15, 103, 1964

208 Sherman, B. S., Maternal-fetal vitamin A levels in hypervitaminosis A-induced teratogenesis, *Anat Rec* , 169, 483, 1971

209 Simon, D J. and Edwards, J. R. G., The distribution of tritiated vitamin A when used as a teratogen in the mouse, *Teratology*, 8, 236, 1973

210 Simon, D J. and Edwards, J R. G., The distribution of tritiated vitamin A when used as a teratogen in the mouse, *Acta Univ Carol Med Monogr* , 56, 273, 1973

211 Kochhar, D M and Kwasigroch, T. E., Demonstration of maternally administered ^{3}H-retinoic acid (vitamin A acid) in mouse conceptus, *Teratology*, 11, 26a, 1975.

212 Kochhar, D M , Transplacental passage of label after administration of ^{3}H-retinoic acid (vitamin A acid) to pregnant mice, *Teratology*, 14, 53, 1976

213 Geelen, J A. G., The localization of vitamin A in the pregnant rat by means of fluorescence microscopy, *Teratology*, 6, 19, 1972

214 Schultz, R. L., Effects of ovariectomy and hypervitaminosis A on lysosomes of the rat conceptus, *Teratology*, 2, 283, 1969

215 Schweichel, J. U., The influence of oral vitamin A doses on interdigital necroses in the limb bud of the rat (day 16 p c), *Teratology*, 4, 501, 1971

216 Schweichel, J U. and Mercker, H. J , The morphology of various types of cell death in prenatal tissues, *Teratology*, 7, 253, 1973

217 Morriss, G. M., An electron microscopic study of the effects of hypervitaminosis A on the maternal-embryonic relationship of the rat at 8 days of pregnancy, *J Reprod Fertil* , 33, 451, 1973

218 Morriss, G. M. and Steele, C E., The effect of excess vitamin A on the development of rat embryos in culture, *J Embryol Exp Morphol* , 32, 505, 1974

219 Morriss, G. M. and Steele, C. E., Comparison of the effects of retinol and retinoic acid on postimplantation rat embryos in vitro, *Teratology*, 15, 109, 1977

220 Thompson, J F and Schweisthal, M. R., Study of closure of the embryonic rat palate in vitro with the effects of certain chemicals, *J Dent Res* , 48, 568, 1969

221 Kochhar, D. M. and Aydelotte, M. B., Limb development in organ culture after transplacental exposure of mouse embryos to retinoic acid, *Teratology*, 4, 233, 1971

222 Kochhar, D. M and Aydelotte, M. B., Susceptible stages and abnormal morphogenesis in the developing mouse limb analysed in organ culture after transplacental exposure to vitamin A (retinoic acid), *J Embryol Exp Morphol* , 31, 721, 1974

223 Kochhar, D M., The use of in vitro procedures in teratology, *Teratology*, 11, 273, 1975

224 Nanda, R., Effect of intra-amniotic injection of vitamin A on rat fetuses, *Teratology*, 9, A31, 1974

225 Nanda, R. and May, D., Influence of amniotic fluid on the in vitro palatal fusion in normal and vitamin A treated rat fetuses, *Teratology*, 9, A31, 1974

226 Kwasigroch, T. E. and Kochhar, D. M., Locomotory behavior of limb bud cells Effects of excess vitamin A in vivo and in vitro *Exp Cell Res* , 95, 269, 1975

227 Morriss, G. M., Abnormal cell migration as a possible factor in the genesis of vitamin A induced craniofacial anomalies, in *New Approaches to the Evaluation of Abnormal Embryonic Development*, Neubert, D and Mercker, H J , Eds , Georg Thieme Verlag, Stuttgart, 1975

228 Wegner, G., Mayer-Rosa, P., and Luz, D., Beitrag zur A-hypervitaminose bei Rattenembryonen Autoradiographische Untersuchungen zur teratogenen Wirkung, *Dtsch Med Wochenschr* , 97, 1071, 1972

229 Schimmelpfennig, K., Baumann, J., and Kaufmann, C., Biochemical and teratological investigations on embryonic glycosaminoglycanes (G A G), *Teratology*, 4, 500, 1971

230 Bernhardt, I. B and Dorsey, D. J., Hypervitaminosis A and congenital renal anomalies in a human infant, *Obstet Gynecol* , 43, 750, 1974

231 Pilotti, G., Ipervitaminosi A in gravidanza e malformazioni dell'apparato urinario nel feto, *Minerva Pediatr* , 27, 682, 1975

232 Pilotti, G. and Scorta, A., Ipervitaminosi A gravidica e malformazioni neonatali dell'apparato urinario, *Minerva Ginecol* , 17, 1103, 1965

233 Morriss, G. M and Thomson, A. D., Vitamin A and rat embryos, *Lancet*, 2, 899, 1974

234 Mounod, R. L , Klein, D , and Weber, F , A propos d'un cas de syndrome de Goldenhar intoxication aigue à la vitamine A chez la mère pendant la grossese, *J Genet Hum.*, 23, 135, 1975

235 Gal, I., Sharman, I M., and Pryse-Davies, J., Vitamin A in relation to human congenital malformations, in *Advances in Teratology*, Vol 5, Woolam, D H M , Ed , Academic Press, New York, 1972

Reprinted from THE ANATOMICAL RECORD
Vol. 189, No. 4, December 1977

Closure of the Neural Tube in the Cephalic Region of the Mouse Embryo

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ABSTRACT In mouse embryos varying in age from 9 to 20 somites the first closure of the neural groove was found to occur in the cervical region. The fusion process gradually proceeded in rhombencephalic direction until it reached a level just caudal to the otic pits. Shortly afterwards the prosencephalic walls fused together independent of the rhombencephalic closure. This prosencephalic fusion process proceeded caudally in the direction of the mesencephalon until it reached the rostral portion of the rhombencephalon. In this region the two independent fusion processes met each other. In addition the prosencephalic fusion proceeded in rostral direction toward the anterior neuropore, which was the last part of the brain vesicles to close. Hence, the closure of the brain vesicles is not a zipper-like process proceeding from the rhombencephalon to the anterior neuropore, but occurs at several places at the same time and proceeds in a rostral as well as in a caudal direction.

At the cellular level considerable differences in the fusion process were found to exist between the various brain vesicles. In the rhombencephalon the first bridge between the two opposing walls was formed by surface ectoderm and neural crest cells. In the mesencephalon single squamous ectoderm and a few neuroepithelial cells established the first contact, whereas in the prosencephalon the apical ends of several neuroepithelial cells fused together to overbridge the gap between the opposing walls. The surface ectoderm cells subsequently covered the neuroepithelial bridge. In the region of the anterior neuropore the fusion was similar to that between the prosencephalic walls, the only difference being that in the anterior neuropore area many more darkly stained particles indicating cell degeneration, were present than in the prosencephalon. It is thus concluded that considerable differences exist in the fusion of the neural walls between the various brain vesicles.

The major morphogenetic event in the development of the central nervous system is the transformation of the neural plate into the neural tube. This invagination process from neural plate to neural groove and subsequently to neural tube has been described by several investigators (Jelínek and Friebová, '66; Langman et al., '66; Schroeder, '70; Burnside, '73; Karfunkel, '74). Considerably less attention, however, has been given to the initial contact and fusion which occur when the tips of the neural walls begin to adhere to each other in the dorsal midline (Marin-Padilla, '70; Schluter, '73; Moran and Rice, '75; Waterman, '76).

In recent years several investigators have examined the fusion process which occurs when two opposing ridges or swellings approach each other and make contact. Hayward ('69) and Hinrichsen and Stevens ('74) noted cell degeneration of the epithelial linings during fusion of the palatine shelves. Greene and Kochhar ('74) suggested that the surface coat found over the epithelial linings of the shelves probably plays an important role in the initial contact. Gaare ('76) investigated the fusion of the medial and lateral nasal swellings and found cell degeneration in the epithelial lin-

Received Feb 4, '77. Accepted July 19, '77

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ings before and at the time that the swellings made their first contact Hay and Low ('72) described the fusion of the endocardial cushions in the heart, and Geeraets ('76) examined the closure of the choroid fissure of the developing eye

In studying the closure of the neural tube in amphibians, Moran and Rice ('75) suggested that the surface coat over the neural walls might play a role in the fusion of the walls When Waterman ('76) studied the closure of the neural tube in the mouse with scanning electronmicroscopy numerous ruffles (lamellapodia) were noted, particularly along the crest of each neural fold They were most prominent near the points of contact and fusion between the folds and were thought to play a role in closure of the neural tube

It was surprising to note that relatively little microscopical work has been performed on fusion of the neural walls, since anencephaly and rachischisis belong to the most frequently seen congenital malformations of the central nervous system Experimental work with teratogenic agents causing anencephaly (Giroud and Martinet, '57, Langman and Welch, '66) and analysis of malformed human embryos (Dekaban, '63, Hannaway and Welch, '70) suggests strongly that this malformation is caused either by a failure of the neural walls to invaginate, or by a lack of fusion of the neural folds in the midline Hence, this study was undertaken to describe the first contact between the tips of the neural walls in the various brain vesicles of the mouse embryo

MATERIALS AND METHODS

Female ICR mice, obtained from the Flow Laboratories (Dublin, Virginia) were mated for a three hour period and subsequently examined for the presence of a vaginal plug The day that a plug was found was considered as day 1 of pregnancy

The pregnant females were sacrificed at different times within a 24 hour period on the ninth and tenth day of gestation Subsequently the embryos were removed from the uterus and the membranes, and fixed in a modified Karnovsky ('65) solution (2% glutaraldehyde, 2% paraformaldehyde in 0 1 M sodium cacodylate buffer, pH 7 3) The embryos were then postfixed for one hour in a 0 1 M cacodylate buffer solution containing 1% OsO_4, and embedded in Araldite Photographs were made of the osmium stained embryos after embedding to examine the extent of the closure at various stages of development After orientation 20 embryos were serially sectioned at 1 μ on a Sorval, Porter Blum MT_2 ultramicrotome Each tenth section was placed on geltatin coated slides, stained with toluidine blue and mounted in Permount

RESULTS

Gross morphological observations

When mouse embryos with 9 to 13 pairs of somites were examined under the dissecting microscope the neural groove was found to be closed in the cervical area (fig 1) In rostral direction the closure had proceeded to a level just caudal to the invaginating otic placodes In the mesencephalon and prosencephalon the neural folds were prominent and separated by a deep groove

During the following hours closure in the rhombencephalic region proceeded slowly in rostral direction and in embryos with 11 to 15 somites it had reached the level of the otic pits (fig 2) The mesencephalic walls were approaching each other, but had not yet fused In the prosencephalon, however, fusion had occurred over a considerable distance and was proceeding in two directions caudally towards the mesencephalon and rostrally towards the anterior neuropore Hence, at this stage of development the mesencephalon and part of the rhombencephalon as well as the most rostral part of the prosencephalon were still open

During further development (16-20 somites) closure in the rhombencephalic region continued slowly in rostral direction, but fusion of the mesencephalic walls proceeded rapidly in the caudal direction (fig 3) These two closing processes met each other in the rostral part of the rhombencephalon At the same time fusion of the prosencephalic walls proceeded towards the anterior rim of the original neural plate This area, which is known as the anterior neuropore, was the last part of the prosencephalon to close (fig 4)

Hence, though it has been generally accepted that closure of the brain vesicles proceeds from the rhombencephalon towards the anterior neuropore as an uninterrupted zipper like process, in mouse embryos the walls of the prosencephalon and mesencephalon fuse independently from those of the rhombencephalon and this fusion process proceeds in caudal as well as in rostral directions

Histological observations

Closure in the rhombencephalic region

When in 11 to 13 somite embryos transverse sections were made from the mesencephalic to the rhombencephalic region, the neural walls were separated in the rostral area, but they approached each other and fused caudally In the most rostral sections the tips of the neural walls contained darkly stained particles (fig 5), indicating cell degeneration In the region where fusion was about to occur, the darkly stained particles were rare In this area the surface ectoderm cells were squamous and curved around the tip of the neural folds (fig 6) Between the surface ectoderm and the neuroepithelial cells was found a group of cells with round to oval nuclei, a rather pale nu cleoplasm and an irregular shape Their orientation and location suggests that they are neural crest cells, migrating from the neuroectodermal junction in a ventro lateral direction The remainder of the neural wall was organized as a pseudostratified neuroepithe lium dividing cells at the lumen, cells with elongated nuclei, oriented perpendicular to the lumen, at the periphery, and small cyto plasmic blebs protruding into the lumen (fig 6)

When the tips of the walls fused an irregularly outlined bridge was formed consisting of large cells, with irregularly shaped nuclei, a pale nucleoplasm and one or two darkly stained nucleoli The cytoplasm was pale and irregular (fig 7) They had the appearance of swollen ectodermal surface cells and neural crest cells The neuroepithelial cells did not appear to participate in the initial contact Once this initial contact had been established the neuroepithelial cells fused in the dorsal midline area and gradually formed a pseudostratified neuroepithelium

Closure in the mesencephalon

In the mesencephalon, where fusion proceeded from rostral to caudal, the tips of the neural walls were characterized by a thin surface ectoderm layer and a well organized neuroepithelium (fig 8) The surface cells were squamous and extended to the tip of the neural wall The pale, irregularly shaped cells seen in the rhombencephalon and thought to be neural crest cells, were absent The first contact between the tips of the walls was established by the surface ectoderm cells and was immediately followed by contact between a few neuroepithelial cells (fig 8) Soon after fusion had occurred the neuroepithelial cells became oriented with their nuclei perpendicular to the lumen thus forming a pseudo stratified epithelium (figs 9, 10)

Fusion in the prosencephalon

In the prosencephalic region the neuroepithelial cells of the opposing walls faced each other with their apical aspects, and the first contact was made by the apical tips of a number of opposing cells (fig 11) Subsequent ly the neuroepithelial cells became reoriented so that the apical aspects faced the lumen and the nuclei were arranged perpendicular to the lumen (fig 12) Contact between the surface ectoderm cells was established only after the neuroepithelium had closed the ventricle Hence, contrary to the rhombencephalic and mesencephalic regions where only a few cells established the first contact, in the prosencephalon a large number of neuroepithelial cells participated in the fusion Before, during and after fusion a few darkly stained particles, indicating cell degeneration, were seen in the tips of the neural walls

Closure of the anterior neuropore

The anterior neuropore closed by fusion of the anterior rim of original neural plate with the two walls of the prosencephalon The most characteristic feature in the tips of the opposing prosencephalic walls was the presence of large numbers of darkly stained particles both in the neuroepithelium and in the ectoderm (fig 13) As far as could be judged from light microscopical observations, most if not all of the particles were located intracellularly

The neuroepithelial cells in the tips of the opposing prosencephalic walls faced each other with their apical ends When the walls began to fuse (fig 14), these apical ends made the first contact The surface ectoderm cells temporarily remained separated Immediately after the first contact had been established, the neuroepithelial cells became arranged with their apical ends to the lumen Finally the surface ectoderm cells overbridged the anterior neuropore (fig 15)

DISCUSSION

Closure of the cephalic part of the neural tube in mammalian embryos is usually described as a continuous zipper-like process

starting in the cervical region and proceeding in rostral direction till it reaches the anterior neuropore This closure mechanism has also been accepted for human embryos, although Streeter ('42), and Bartelmez and Dekaban ('62) have shown that the anterior neuropore is also closed by a fusion process proceeding in opposite (rostro caudal) direction From our observations it is evident that in the mouse the fusion of the neural walls is not an unin terrupted process, but occurs in different re gions at the same time A somewhat similar observation was made by Edwards ('68) and Christie ('69) in rat embryos and by Keyzer ('72) and Shenefelt ('72) in hamster embryos Waterman ('76) also demonstrated that fusion occurs simultaneously in the prosencephalon and in the hindbrain region The last opening to close was at the level of the mesencephalon Hence, it is likely that in several mammalian species the prosencephalon is closed while part of the rhombencephalic mesencephalic region is still open

The observation that the prosencephalon closes before the mesencephalon makes it pos sible to explain the different types of brain malformations observed when mammalian embryos are treated with teratogens When Giroud and Martinet ('57) and Giroud ('60) ex amined litters from pregnant rats given ex cess vitamin A, fetuses with anencephaly, with an unclosed mesencephalon, with men-ingo-encephalocele and meningocele in the mesencephalic region were found within the same litter The authors therefore suggested that the various types of brain malformations were probably the result of one primary dis turbance absent or insufficient closure of the neural tube Since the prosencephalon closes before the mesencephalon and considerable differences in development exist between em-bryos within the same litter, it is likely that the youngest, least developed, embryos with an open neural groove in the cephalic region will show anencephaly as a result of the tera togen treatment Those embryos, which are somewhat more advanced and in which the prosencephalon is closed at the time of treat-ment, will show mesencephalic abnormalities The most advanced embryos will not show any gross brain abnormalities since all vesicles are closed at the time of treatment Hence, the different types of brain abnormalities seen after treatment with teratogens are probably due to temporal and spatial differences in the closure process between the embryos

The temporal and spatial differences in the closure process, however, cannot explain why some teratogenic substances cause anen-cephaly and no rachischisis, while other agents cause various degrees of rachischisis without brain abnormalities It is therefore probable that the differences observed be tween the fusion processes of the various brain vesicles at the cellular level also play an important role In the caudal part of the rhombencephalon contact between the oppos-ing neural walls is made by surface ectoderm and neural crest cells A similar type of fusion was described in the spinal cord of the chick embryo (Virgilio et al , '67) and in that of the hamster (Marin Padilla, '70) In the prosen-cephalon the first contact is made by rows of opposing neuroepithelial cells In the mesen-cephalon fusion is most delicate and is estab-lished by a few squamous surface ectoderm and neuroepithelial cells It seems therefore possible that the various fusion processes may have a different susceptibility when exposed to teratogenic agents Some teratogenic sub-stances may interfere particularly with neu-ral crest cell fusion, others may have a special effect on fusion between neuroepithelial cells

Comparing the data from this study with fusion processes already described in other re-gions of the embryo, the variety of fusion mechanisms is striking Fusion of the palatine shelves can only be accomplished after degen-eration of the epithelial linings (Hayward, '69, Hinrichsen and Stevens, '74) Before the endocardial swellings are able to fuse the cells on the opposing surfaces are transformed into connective tissue cells (Hay and Low, '72) The choroid fissure is bridged only after the ap-proaching basal laminae have disappeared and some cell degeneration has occurred (Geeraets, '76) The fusion of the medial and lateral nasal swellings is accompanied by de-generation of the covering epithelial linings (Gaare, '76) However, in fusion of the neural walls no cellular components or basal laminae are interposed between the presumptive fusion areas and, indeed, in most fusion areas cell death is absent or negligible The excep-tion is found in the prosencephalon and par-ticularly in the region of the anterior neu-ropore where many darkly stained particles were observed Glucksmann ('51), who noted similar particles in the tips of the neural walls close to the anterior neuropore, interpreted these bodies as indications of physiological cell death Schluter ('73), who examined the

same area with the electron microscope suggested that the particles were phagocytosed debris from degenerated fragmented cells Although we agree that the darkly stained particles are indications of physiological cell death, we do not think that this cell death is related to the fusion process such as described in the fusion of the palatal shelves and nasal swellings In the region of the anterior neuropore cell death is probably the result of a morphogenetic mechanism necessary to remodel the neural walls

ACKNOWLEDGMENTS

This work was supported by Grants NS 06188 and DE 07037 to Doctor Jan Langman and by an IBRO Fellowship Neurobiology from UNESCO and the Government of the Netherlands to Doctor Jan A G Geelen

We wish to thank Doctor J D Gaare for providing us with helpful suggestions and some of the experimental material We are also indebted to Miss Linda Chia for helping us with the technical procedures, and Mrs Susan Seiler for typing the manuscript

LITERATURE CITED

Bartelmez G W , and A S Dekaban 1962 The early development of the human brain Carnegie Inst Wash Publ 621 Contribs Embryol , *37* 13 32

Burnside, B 1973 Microtubules and microfilaments in amphibian neurulation Am Zool , *13* 989 1006

Christie, G A 1964 Developmental stages in somite and post somite rat embryos based on external appearance, and including some features of the macroscopic development of the oral cavity J Morphol , *114* 263 286

Dekaban, A S 1963 Anencephaly in early human embryos J Neuropathol Exp Neurol , *22* 533 548

Edwards, J A 1968 The external development of the rabbit and rat embryo In Advances in Teratology Vol 3 D H M Wollam, ed , Academic Press, New York, pp 239 263

Gaare, J D 1976 Cell degeneration during the fusion of the nasal processes in mice Anat Rec , *184* 407

Geeraets, R 1976 An electronmicroscopic study of the closure of the optic fissure in the golden hamster Am J Anat , *145* 411 432

Giroud, A 1960 Causes and morphogenesis of anencephaly In CIBA–foundation Symposium on Congenital Malformations G E W Wolstenholme and C M O'Connor, eds Little, Brown and Co , Boston

Giroud A , and M Martinet 1957 Morphogenese de l' anencephalie Arch Anat Microsc Morphol Exp , *46* 247 264

Glucksmann, A 1951 Cell deaths in normal vertebrate ontogeny Biol Rev *26* 59 86

Greene, R M , and D M Kochhar 1974 Surface coat on the epithelium of developing palatine shelves in the mouse as revealed by electronmicroscopy J Embryol Exp Morphol , *31* 683 692

Hannaway, J , and G W Welch 1970 Anencephaly A review and interpretation in terms of modern experimental embryology Dis Nerv Syst , *31* 527 533

Hay, D A , and F L Low 1972 The fusion of dorsal and ventral endocardial cushions in the embryonic chick heart, a study in fine structure Am J Anat , *133* 1 24

Hayward A F 1969 Ultrastructural changes in the epithelium during fusion of the palatal processes in rats Arch Oral Biol *14* 661 678

Hinrichsen C F L and G S Stevens 1974 Epithelial morphology during closure of the secondary palate in the rat Arch Oral Biol , *19* 969 980

Jelinek, R , and Z Freibova 1966 Influence of mitotic activity on neurulation movements Nature, *209* 822 823

Karfunkel, P 1974 The mechanism of neural tube formation Int Rev Cytol , *38* 245 271

Karnovsky, M J 1965 A formaldehyde glutaraldehyde fixative of high osmolality for use in electronmicroscopy J Cell Biol , *27* 137A 138A

Keyzer, A 1972 The development of the diencephalon of the Chinese Hamster Acta Anat (Basel), Suppl 59

Langman, J , Guerrant and B Freeman 1966 Behavior of neuroepithelial cells during closure of the neural tube J Comp Neur *127* 399 411

Langman, J , and G W Welch 1966 Effect of vitamin A on development of the central nervous system J Comp Neur , *128* 1 16

Marin Padilla, M 1970 The closure of the neural tube in the Golden Hamster Teratology *3* 39 46

Moran, D , and R W Rice 1975 An ultrastructural examination of the role of cell membrane surface coat material during neurulation J Cell Biol , *64* 172 181

Schluter, G 1973 Ultrastructural observations on cell necrosis during formation of the neural tube in mouse embryos Z Anat Entwicklungsgesch , *141* 251 264

Schroeder, T E 1970 Neurulation in *Xenopus laevis* An analysis and model based upon light and electronmicroscopy J Embryol Exp Morphol , *23* 427 462

Shenefelt, R E 1972 Morphogenesis of malformations in hamsters caused by retinoic acid relation to dose and stage at treatment Teratology, *5* 103 118

Streeter, G L 1942 Developmental horizons in human embryos Description of age group XI, 13 20 somites, and age group XII, 21 29 somites Carnegie Inst Wash Publ 541, Contribs Embryol , *30* 211 245

Virgilio, G di, N Lavenda and J L Worden 1967 Sequence of events in neural tube closure and the formation of neural crest in the chick embryo Acta Anat (Basel), *68* 127 146

Waterman, R E 1976 Topographical changes along the neural fold associated with neurulation in the hamster and mouse Anat Rec , *146* 151 172

PLATE 1

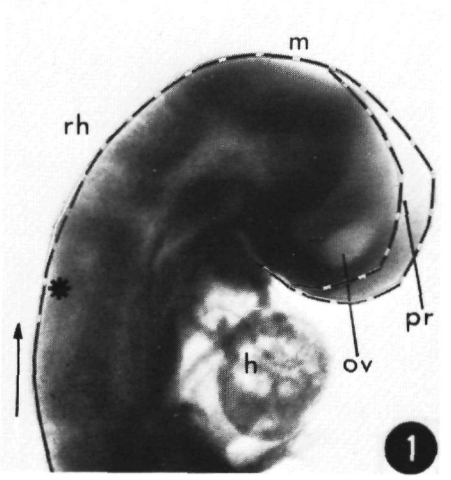
EXPLANATION OF FIGURES

1 Photograph of a 9-somite mouse embryo The closed part of the neural tube is indicated by a solid line, the open part by dotted lines The arrow indicates the direction in which the fusion process proceeds. Note: the otic placode (*); rhombencephalon (rh), mesencephalon (m); optic vesicle (ov), prosencephalic walls (pr) and the heart (h). × 75.

2 Photograph of an 11-somite embryo The rhombencephalic closure has proceeded in rostral direction to the otic pit. The walls of the prosencephalon have fused over a considerable distance and this fusion process is proceeding in rostral as well as in caudal direction (arrows). Note the otic pit (*). × 75.

3 Photograph of a 16-somite embryo. The rhombencephalic closure has proceeded beyond the level of the otic pit (*). Fusion has also proceeded from the prosencephalon to the mesencephalon, which at this stage is almost entirely closed. In rostral direction fusion has proceeded to the anterior neuropore (a np). × 75.

4 Photograph of a 20-somite embryo. The cephalic part of the neural tube is completely closed × 75.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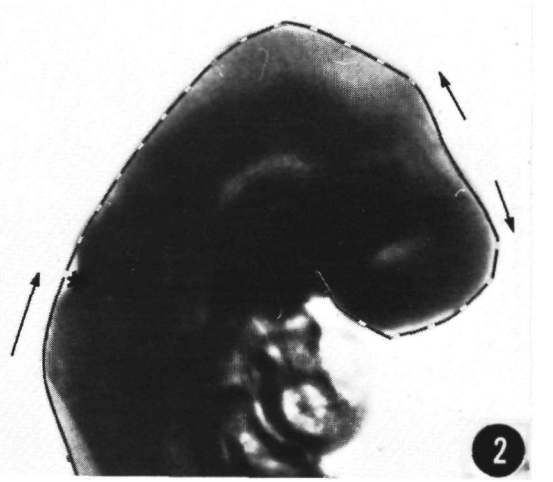
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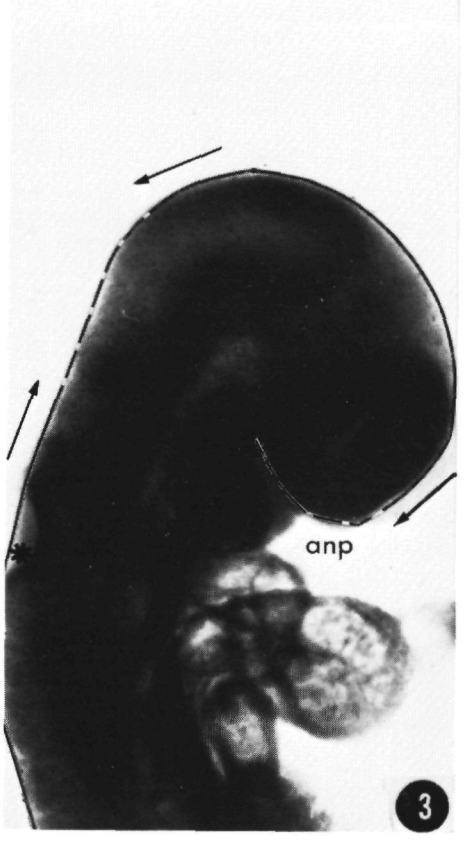
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PLATE 2

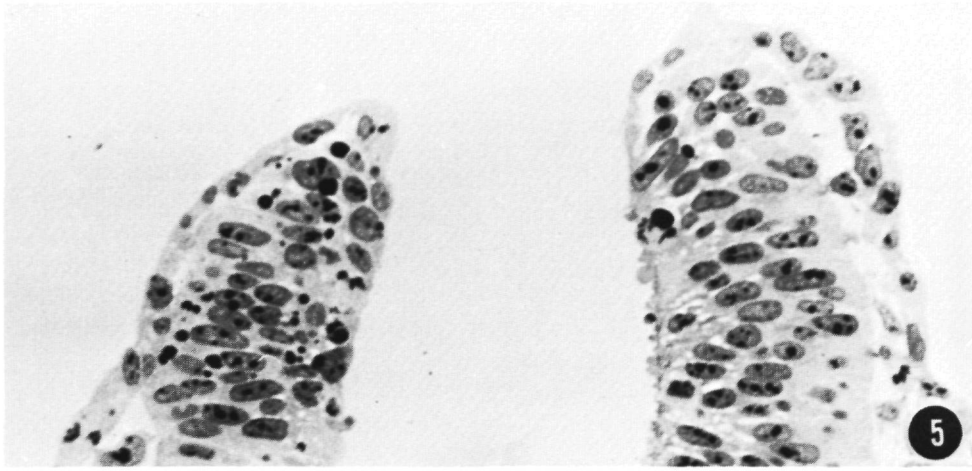
EXPLANATION OF FIGURES

Closure of the rhombencephalon

5 Transverse section through the caudal part of the rhombencephalon of a 12 somite embryo The surface ectoderm cells cover the tip of the neural walls In the region of the tip of the neural wall the surface is smooth, in the neuroepithelial region it shows many irregular cytoplasmic protrusions Darkly stained particles are present in the tip of the neural wall × 500

6 Transverse section through the rhombencephalon slightly more caudal than represented in figure 5 The surface ectoderm covers the tip of the neural wall, which is made up of cells with round to oval nuclei, considered to be neural crest cells Note the neuroepithelial cells in the remainder of the wall and the irregular luminal surface × 500

7 Transverse section through the rhombencephalon during the fusion process, slightly more caudal than the previous sections The bridge between the two neural walls is formed by large pale cells with irregularly shaped nuclei, and consists of swollen surface ectoderm and neural crest cells × 500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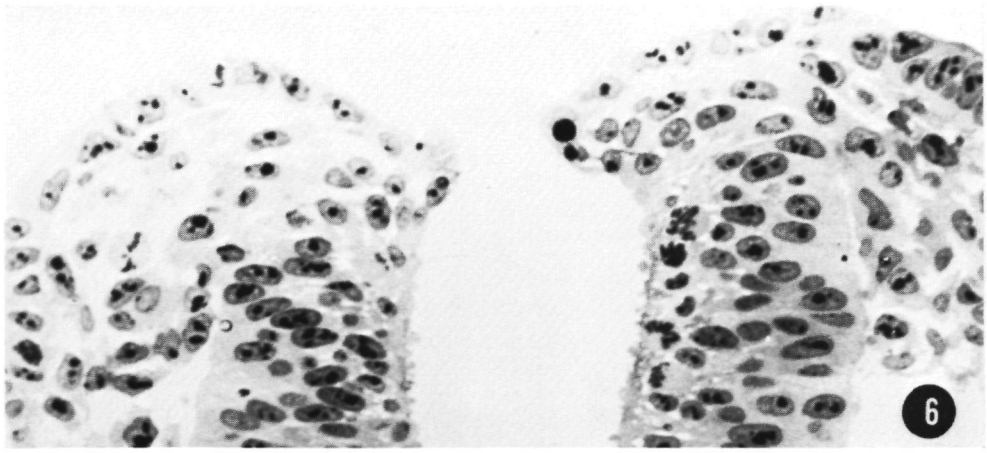
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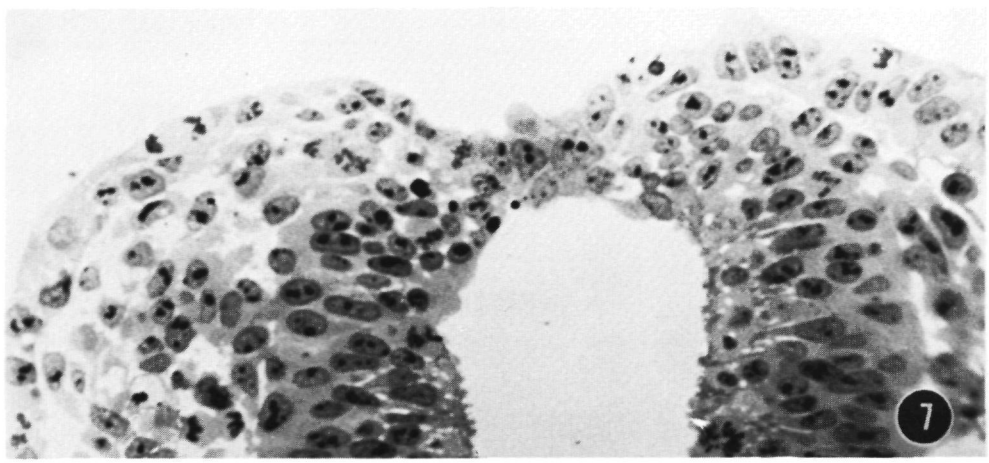
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PLATE 3

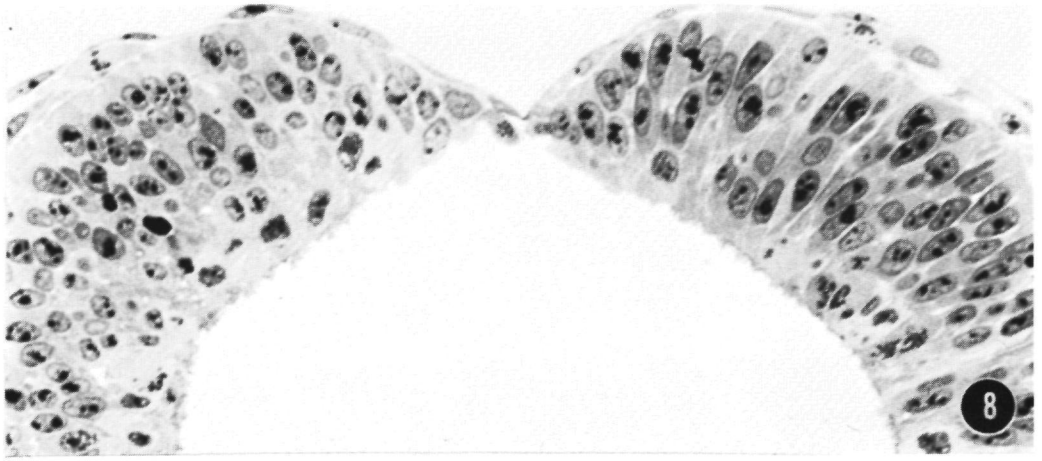
EXPLANATION OF FIGURES

Closure of the mesencephalon

8 Transverse section through the mesencephalon of a 14 somite embryo The squamous surface ectoderm and neuroepithelial cells are making contact × 500

9 Transverse section through the mesencephalon slightly more rostral than in figure 8 The neuroepithelial cells in the bridge area are already oriented with their nuclei perpendicular to the lumen × 500

10 Similar section as in figure 9, but slightly more rostral Fusion has been established and the neuroepithelial layer in the fusion area is almost complete × 500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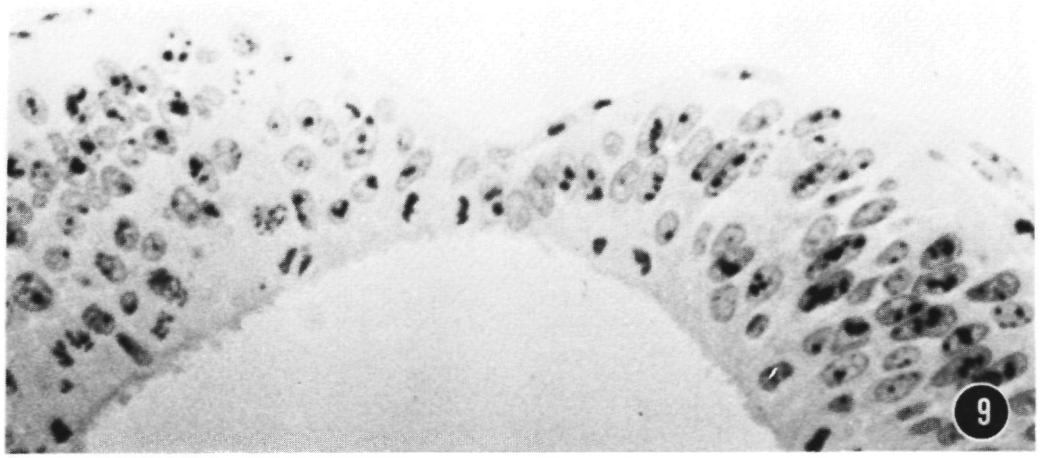
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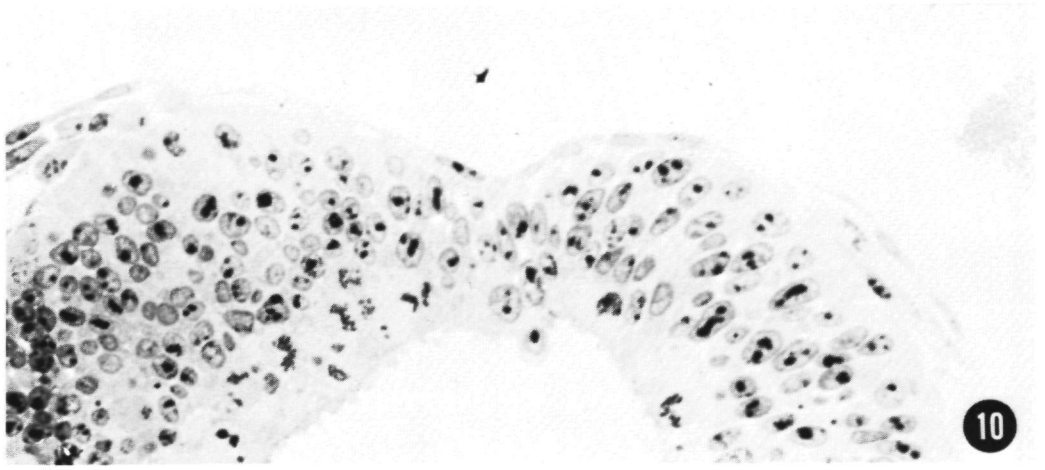
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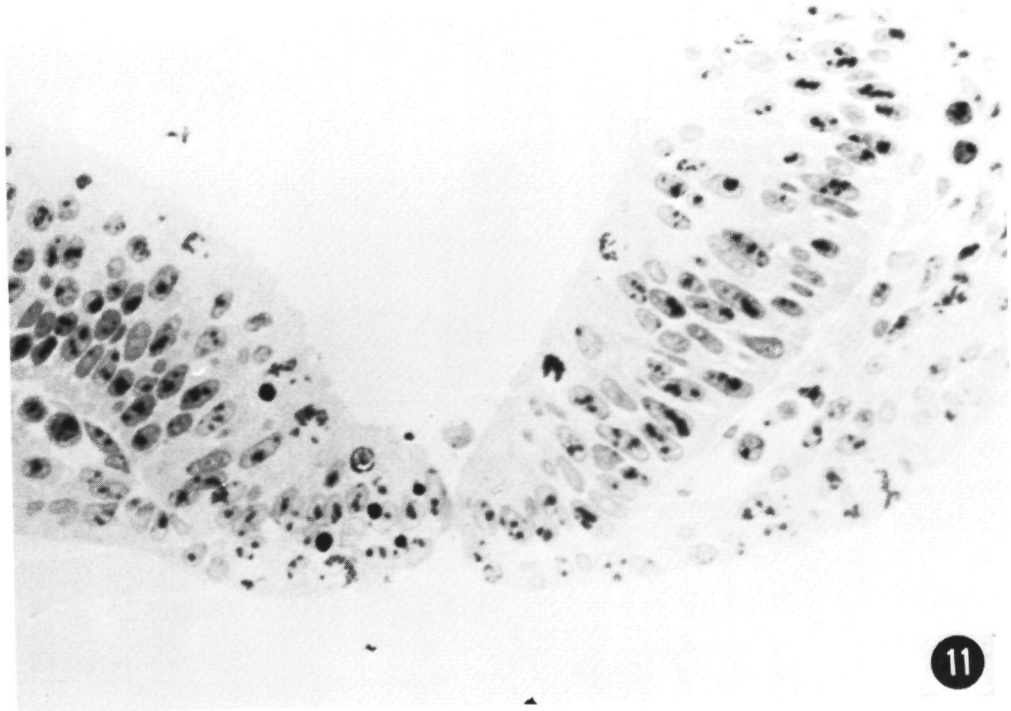
PLATE 4

EXPLANATION OF FIGURES

Closure of the prosencephalon

11 Transverse section through the tips of the neural walls in the prosencephalon of a 14-somite embryo The neuroepithelial cells of the two opposing walls have just established contact with each other The ectodermal cells are still separated by a wide gap. × 500.

12 Similar section as in figure 11, but slightly more caudal The ectodermal surface cells have fused with each other, and most of the neuroepithelial cells are oriented with their apical surface toward the lumen × 500.



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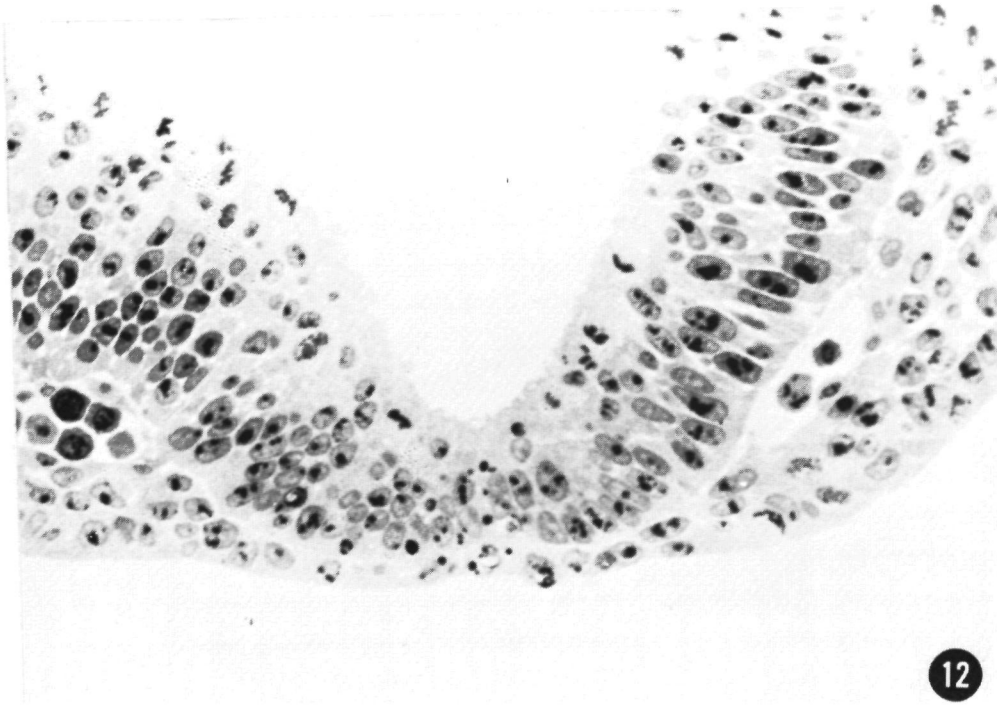

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PLATE 5

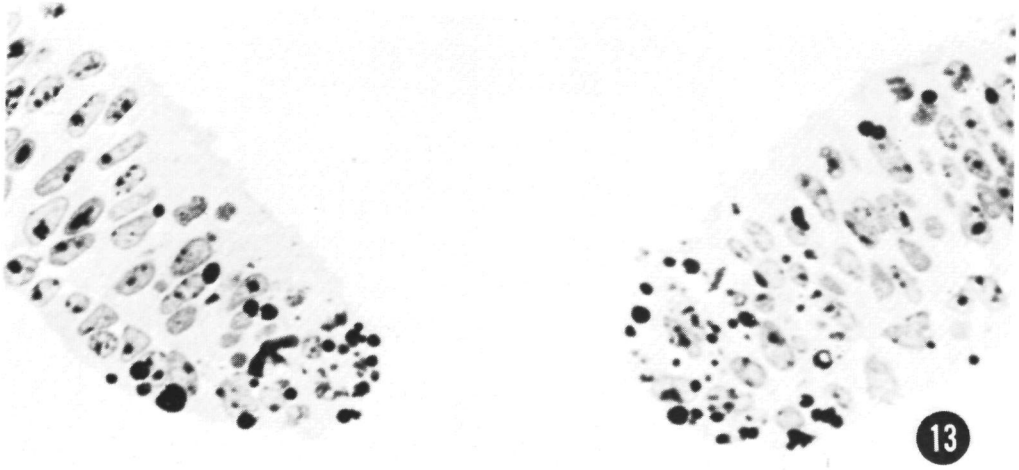
EXPLANATION OF FIGURES

Closure of the anterior neuropore

13 Transverse section through the region of the anterior neuropore in a 12-somite embryo. The tips of the neural walls are characterized by many darkly stained particles, possibly representing debris of degenerated cells × 500.

14 Similar section as in figure 13, but slightly more rostral (closer to the primitive oral cavity). The neuroepithelial cells of the opposing wall face each other with their apical ends and have just established contact. × 500

15 Similar section as in figure 14, but slightly more rostral. The neuroepithelial cells in the fusion area have become oriented with their apical ends facing the lumen. The surface ectoderm cells are about to make contact with each other. × 500.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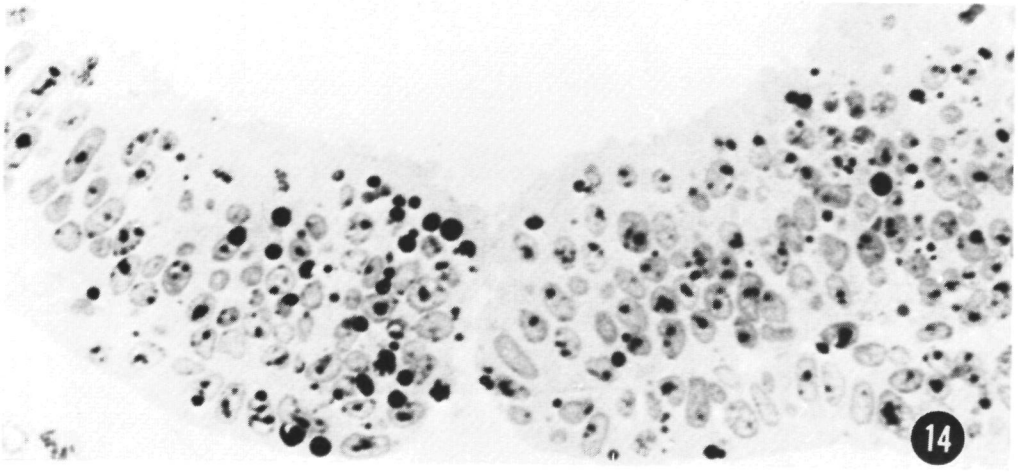
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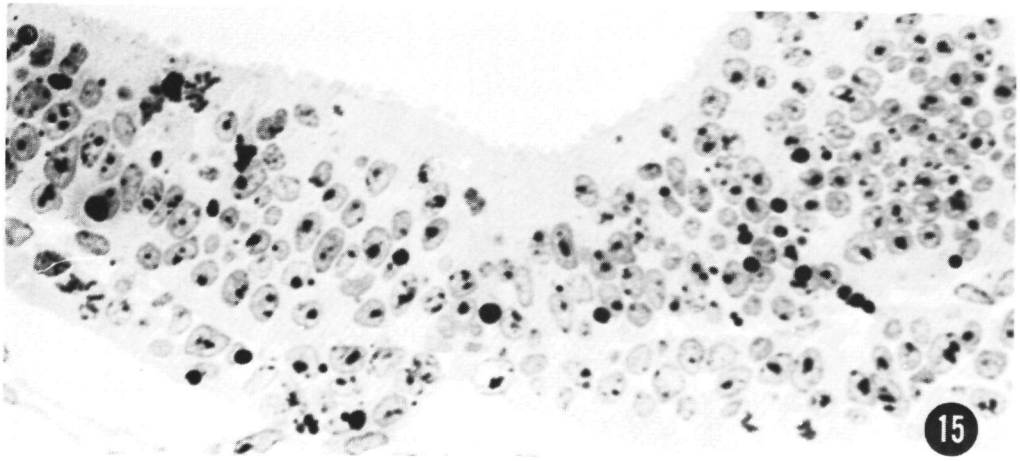
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Anat Embryol 156 73 88 (1979)

Anatomy
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Ultrastructural Observations on Closure of the Neural Tube in the Mouse

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Summary. The fusion of the neural walls in the cephalic part of mouse embryos varying in age from 9 to 20 somites was examined with the electron microscope In the rhombencephalic region the rim of the neural wall was formed from outside inward by ectodermal surface cells, a row of flattened cells without surface projections and neuroepithelial cells At the junction of the surface ectoderm and the flat cells were seen large projections containing a cytoplasmic matrix without organelles and previously referred to as "ruffles" The initial contact between the walls was made by the large cytoplasmic arms and numerous finger-like projections interdigitating with similar projections from the opposite wall The projections originated from the surface ectoderm and possibly neural crest cells During further fusion the surface ectoderm cells formed dense membrane specializations, thus establishing a firm contact.

The initial contact in the mesencephalon was formed by extensions from the surface ectoderm and was followed by the formation of specialized membrane junctions, as seen between the surface ectoderm in the rhombencephalon. The neuroepithelial cells facing the gap between the neural walls with their apical ends made contact with the cells from the opposing wall by numerous finger-like projections but membrane specializations failed to develop

The closing mechanism in the prosencephalon and anterior neuropore regions differed from the previous areas in that the initial contact was established by the neuroepithelial cells Only after this contact had been formed did the surface ectoderm cells close the gap In contrast with the other areas many phagocytosed particles were seen in the prosencephalon and

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in the region of the anterior neuropore Many particles from degenerated cells were found inside healthy surrounding cells Some of these particles contained nuclear material and cytoplasmic organelles

Key words: Closure – Development – Mammalian embryo – Neural tube – Ultrastructure

Introduction

From recent studies on closure of the neural tube in mouse embryos it has become evident that fusion of the neural walls does not proceed from the rhombencephalon to the anterior neuropore in a zipper-like fashion In the rhombencephalon and prosencephalon fusion of the neural walls proceeds simultaneously (Geelen and Langman, 1977) Similar observations were made in the rat by Edwards (1968) and Christie (1964) and in the hamster by Keyser (1972) and Shenefelt (1972) With scanning electron microscopy Waterman (1976) similarly demonstrated that fusion occurs simultaneously in the hindbrain and in the forebrain

Examining the cells involved in the initial contact between the opposing neural walls, it was found that in the rhombencephalon surface ectoderm and probably neural crest cells were the first ones to make contact, in the prosencephalon and particularly in the region of the anterior neuropore, the neuroepithelial cells were involved in the fusion process in the mesencephalon, the first contact was mainly made by surface ectoderm cells (Geelen and Langman, 1977)

A number of investigators have examined the neurulation process in amphibian and chick embryos with the scanning electron microscope (Tarin, 1971, Gouda, 1974, Barson and Portch, 1974, and Portch and Barson, 1974), but little work has been performed on the closure process of the neural tube in the mammalian embryo (Waterman, 1975a, b, 1976) When Waterman studied the cellular morphology along the rims of the neural folds before and after fusion in hamster and mouse embryos, a narrow band of flattened cells was found between the surface ectoderm and the neural ectoderm regions In the mouse numerous membranous "ruffles" were seen at the junction of the surface ectoderm and the so-called flat cells and these ruffles were thought to establish the first contact between the opposing folds Since scanning electronmicroscopy provides mainly surface characteristics, it was difficult to determine how contact between the cells in the deeper layers was established

Only few studies have been performed on closure of the neural tube with transmission electronmicroscopy (Schroeder, 1970, Lofberg, 1974, Bancroft and Bellairs, 1975, Schluter, 1973) Schluter examined the cells of the neural tube in the mouse embryo before and after closure, but focused his attention on the role of cell death Bancroft and Bellairs (1975) noted in the chick embryo that at the time of fusion some threads, probably formed by the fusion of two projections, were connecting the opposing folds Microvilli and other cytoplasmic processes appeared to be touching cells in the opposite neural fold, but cell junctions were not observed

This study was undertaken to examine with the transmission electronmicroscope the manner by which cellular contact between the approaching folds is established and whether membrane specializations are formed.

Materials and Methods

Female ICR mice from Flow Laboratories (Dublin, Va.) were mated from 9–12 A.M. and subsequently examined for the presence of vaginal plugs. The day on which a plug was found, was considered as day 1 of gestation.

The pregnant females were sacrificed on day 10 at 10 A.M. It is known that at this stage of development embryos at different stages of neural tube closure can be found in one litter (Geelen and Langman, 1977). Therefore this stage of development is particularly suitable to examine the first contact and subsequent fusion between the opposing walls. After the embryos were removed from the uterus and membranes, they were fixed for one hour in modified Karnovsky (1965) solution (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3). The tissue was then post-fixed for one hour in 0.1 M cacodylate buffer containing 1% OsO_4 and after dehydration in ethanol embedded in Epon 812. Embryos in which the prosencephalon, mesencephalon and rhombencephalon were partly closed were selected for sectioning. After orientation they were cut at 1 μm on a Sorvall, Porter-Blum MT2 ultramicrotome. As soon as one of the fusion areas between the neural walls was reached, 300–600 Å sections were cut with a diamond knife. The thin sections were subsequently stained with uranyl acetate and lead citrate and examined with a RCA EMU 3 electron-microscope. In this manner sections of the fusion process in the rhombencephalon, mesencephalon, prosencephalon and the anterior neuropore were obtained.

Results

Closure of the Rhombencephalon. In the rhombencephalon region a single layer of ectoderm cells covered the outer surface of the embryo up to the rim of the neural wall (Fig. 1). These surface ectoderm (SE) cells were characterized by microvilli projecting from the surface. Immediately over the tip of the neural wall and facing the lumen of the neural groove one or sometimes two flat attenuated epithelial cells (FE) were seen (Figs. 1 and 2). Over most of their surface they had few if any cytoplasmic projections extending into the lumen of the groove. At the junction of the flat epithelial cell with the surface ectoderm, however, were always noted thick projections (R) containing cytoplasmic matrix without organelles (Figs. 1 and 2). These unusually large projections (in SEM studies referred to as ruffles) were sometimes cup-shaped, but more frequently had the appearance of multiple fingerlike extensions. They were greatly different from the microvilli on the surface ectoderm. In some instances the ruffles were seen to originate from the flat epithelial cells (Fig. 2) and in other instances from the surface ectoderm cells (Fig. 1). Deeper in the neural groove the wall was formed by the apical ends of the neuroepithelial cells (NE), which were always connected to each other by typical tight junctions. Cytoplasmic blebs containing ribosomes, mitochondria and rough endoplasmic reticulum protruded from the apical surface and formed the luminal surface (Fig. 3).

In sections closer to the fusion area long cytoplasmic arms extended into the gap between the two opposing rims (Fig. 4). The actual contact was made by numerous finger-like projections interdigitating with similar projections from

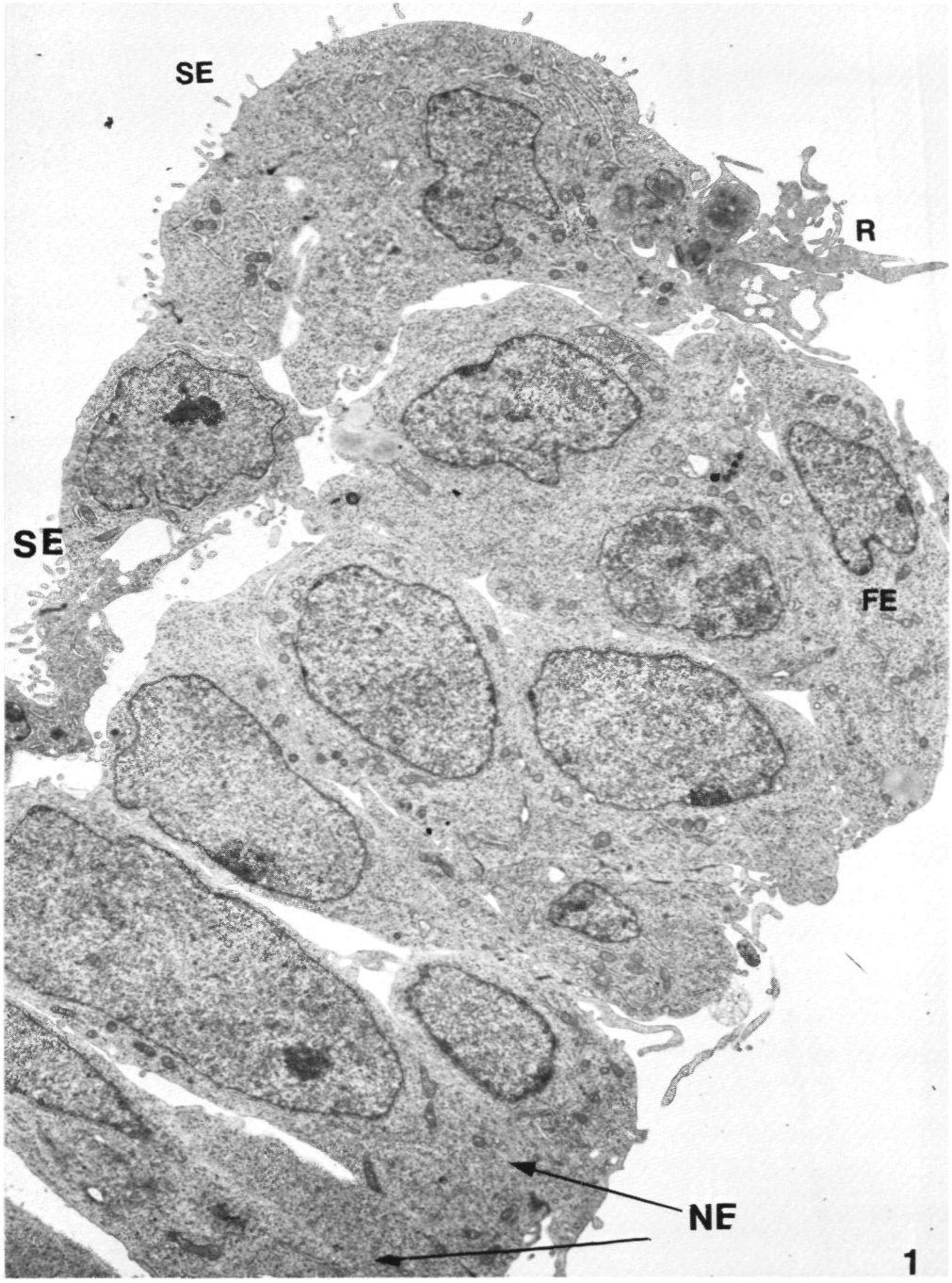
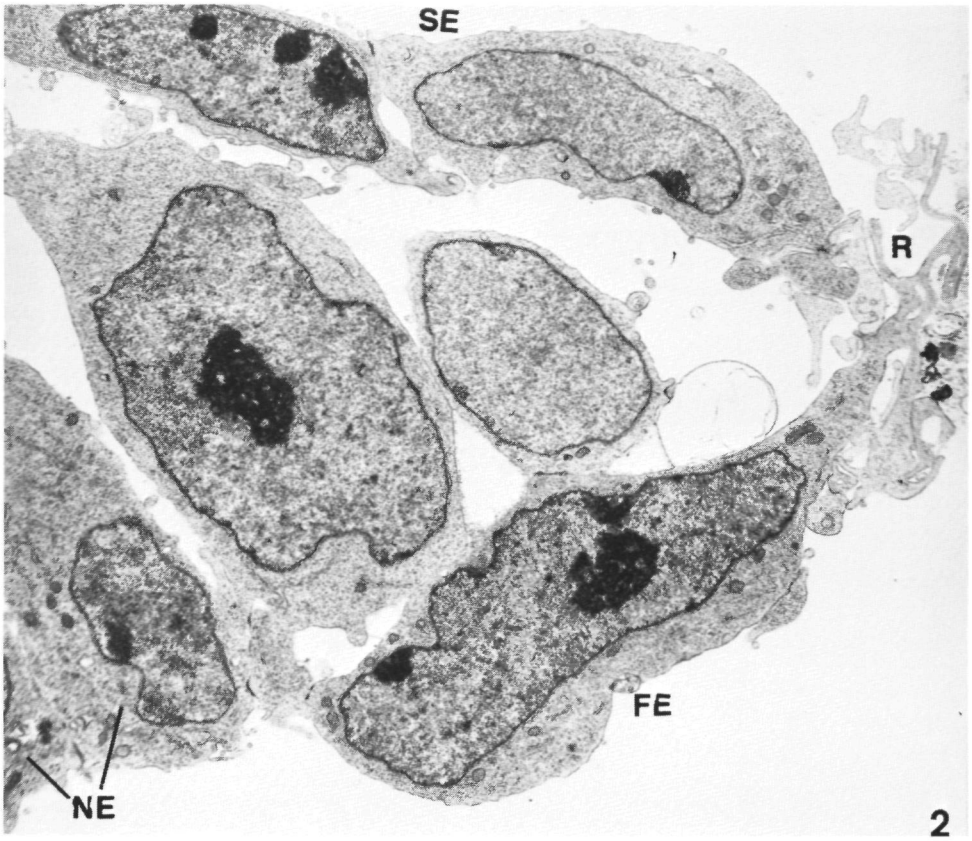


Fig. 1. Transverse section through the rhombencephalon prior to fusion. The cellular components of the rim of one of the neural walls are shown. Note the surface ectoderm (*SE*), the flat epithelial (*FE*) cell and the neuroepithelial (*NE*) cells. A prominent cytoplasmic extension, a so-called ruffle (*R*) is present at the junction of the surface ectoderm and the flat epithelial cell. 4,500×



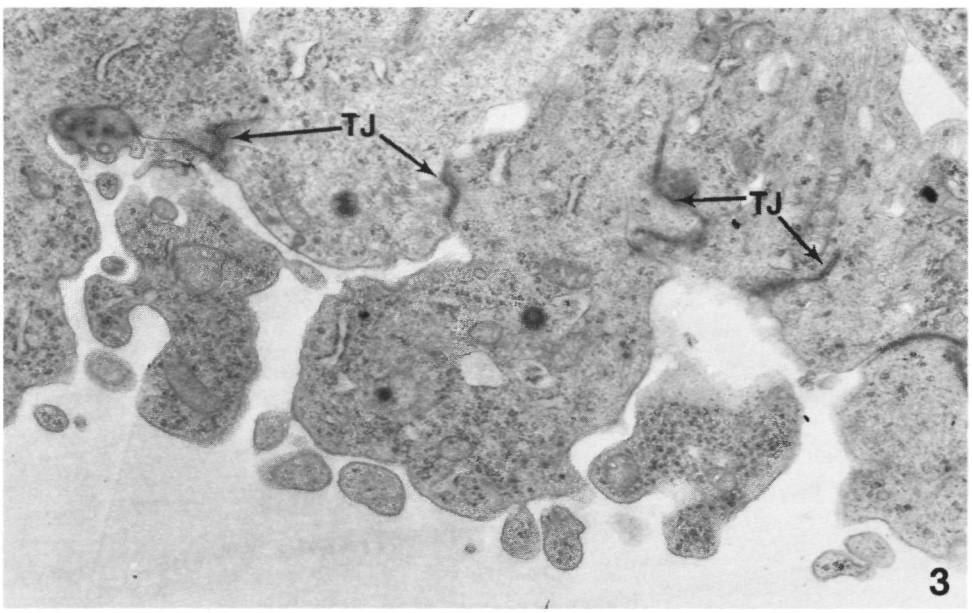


Fig. 2. Rim of neural wall in rhombencephalic region showing surface ectoderm (*SE*) cells, a flat epithelial cell (*FE*) and neuroepithelial cells (*NE*). Note that the cytoplasmic extension, referred to as ruffle (*R*), seems to originate from the flat cell. 4,600×

Fig. 3. Cytoplasmic blebs protruding from the apical ends of the neuroepithelial cells. The protrusions contain polyribosomes, mitochondria and rough endoplasmic reticulum. Note the tight junctions (*TJ*) between the cells at the apical ends. 12,000×

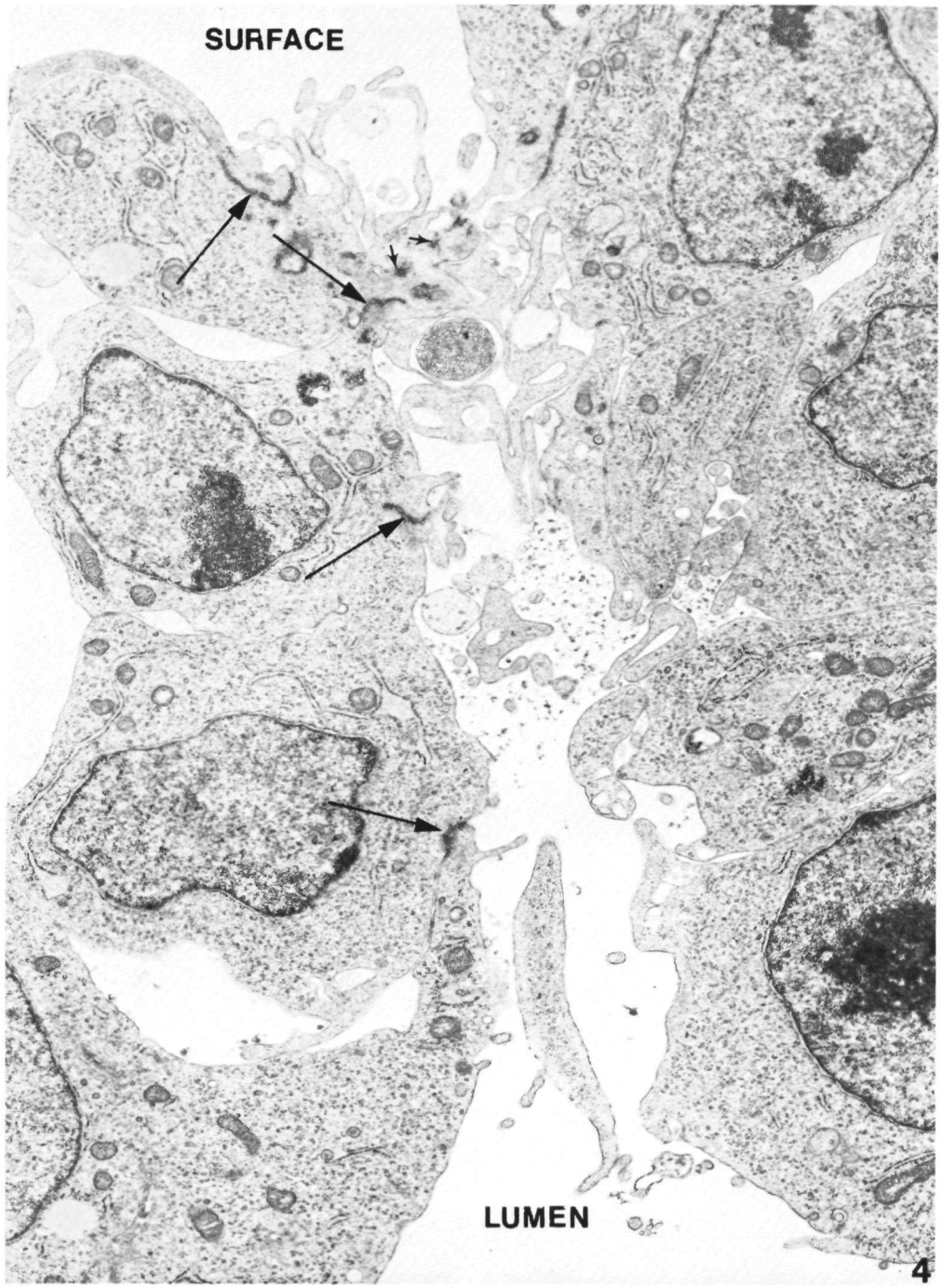


Fig. 4. Initial contact between the opposing rhombencephalic walls. Numerous extensions protrude in the gap between the two rims. They are particularly evident in the region close to the surface where previously the ruffles were seen. Note the small electron dense areas (arrowheads) between the projections of the two opposing walls. Also note the contact areas (arrows) between adjacent cells on the neural rim. 11,000 ×

the opposite wall Some of these projections originated from surface ectoderm cells but others from cells located immediately below the surface These latter cells did not resemble the attenuated flat epithelial cells seen before the closure, nor did they resemble the elongated neuroepithelial cells They probably represent neural crest cells Although distinct surface specializations were visible between adjacent cells in the neural wall (Figs 3 and 4), in the contact area between the opposing walls only a few very small electron dense areas were observed (Fig 4) Slightly further into the fusion area both the surface ectoderm cells and the cells immediately under the surface (possibly neural crest cells) established broad contact with similar cells from the opposite wall, but no elaborate junctions between opposing cells were seen (Fig 5) Still further into the fusion area the neuroepithelial cells contacted each other and became aligned with their apical ends towards the lumen Hence, the first contact in the rhombencephalic region was established by surface ectoderm, this was followed by contact between (possibly) neural crest cells and finally by contact between the neuroepithelial cells

Closure of the Mesencephalon On approaching the fusion area in the mesencephalon, the cellular composition of the rim of the neural wall changed The flat attenuated epithelial cells were absent, and the surface ectoderm cells now extended over the tip of the rim to make direct contact with the neuroepithelial cells

In the fusion area many extensions of the surface ectoderm interdigitated with similar structures from the opposite side, a picture greatly similar to that observed in the rhombencephalon This initiale contact was soon followed by a stage characterized by more extensive and elaborate junctions than seen in other areas (Fig 6) The precise nature of these junctions was difficult to determine but adjacent to them were usually found areas containing a dark filamentous material (Fig 6) Hence, the initial contact in the mesencephalon was established by surface ectoderm cells and this contact seemed to be more elaborate than in other areas. Subsequently the neuroepithelial cells of the opposing walls made contact by large cytoplasmic extensions, but failed to develop any membrane specializations comparable to those seen between the surface ectoderm cells Finally the neuroepithelial cells became oriented perpendicular to the lumen, and the regular membrane specializations developed at their apical ends (Fig 3)

Closure of the Anterior Neuropore. In the region of the anterior neuropore the neural walls were initially separated by a relatively wide gap The rims were covered by surface ectoderm, which was in direct contact with the neuroepithelial cells further down in the gap Closer to the fusion area the surface ectoderm receded and the first contact between the opposing walls was established by the neuroepithelial cells which faced each other with their apical ends (Fig. 7). Numerous small processes protruded from the cells (Fig 8) At their distal ends they contained some flocculent material, but with the exception of some ribosomes, other organelles were lacking No distinct membrane specializations were seen between processes from cells of the opposing walls Hence, although

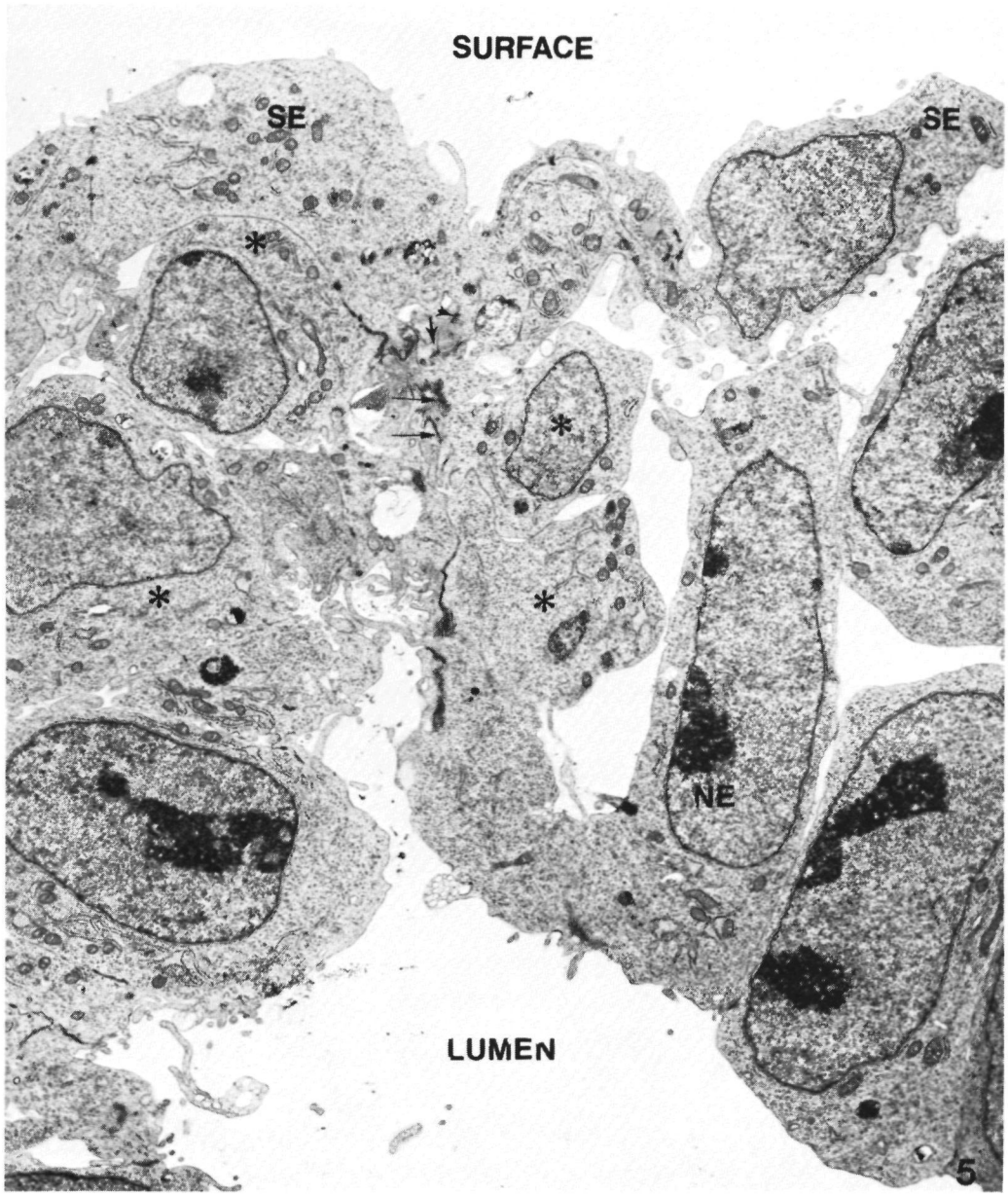


Fig. 5. Section slightly further into the area of fusion of the rhombencephalic walls as in Fig. 4. At the surface contact seems firmly established and a number of small electron dense areas (arrowheads) are visible. In the region just below the surface numerous cytoplasmic extensions occupy the space between the two walls and numerous small cytoplasmic extensions interdigitate with each other. Note also the neuroepithelial cells (*NE*). The cells immediately beneath the surface ectoderm probably represent neural crest cells (*). 5,000×

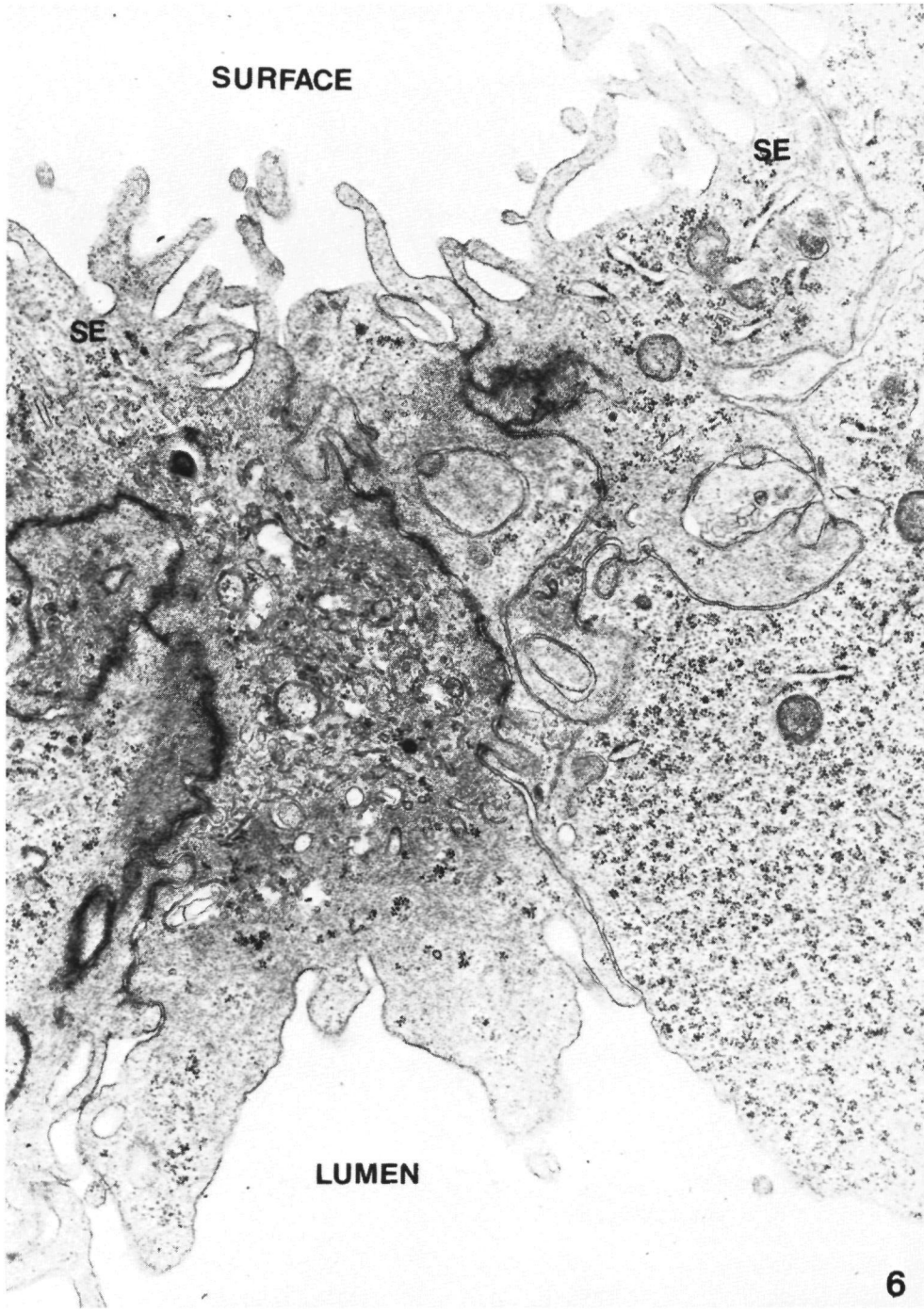


Fig. 6. Transverse section through the fused region of the mesencephalon. The surface ectoderm (*SE*) cells, characterized by numerous microvilli, have established firm contact between the two rims. In the fused area several electron dense junctional complexes extending over considerable distance are visible. Note the filamentous material beneath the junctions. 18,000 ×

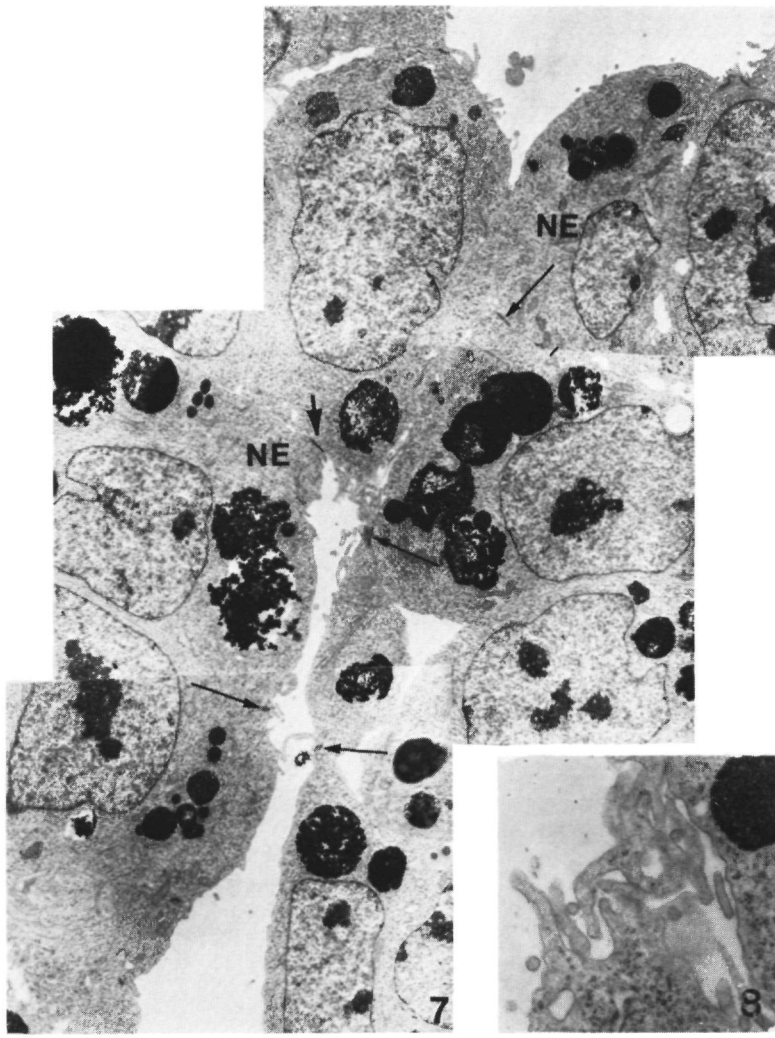


Fig. 7. Transverse section through the walls of the anterior neuropore showing the beginning of fusion between neuroepithelial cells (*NE*) of the opposing walls. Note the junctions (arrows) between adjacent neuroepithelial cells and the numerous cytoplasmic processes extending in the gap. Most neuroepithelial cells contain phagocytosed particles consisting of degenerated debris. Degenerating cells in the process of condensation and fragmentation are absent. 3,000×

Fig. 8. Magnification of cytoplasmic extensions arising from the apical ends of the neuroepithelial cells. They contain some flocculent material and ribosomes. 12,000×

distinct membrane specializations were found between adjacent neuroepithelial cells on either side of the groove, no such junctions were observed between the apical ends of opposing cells (Figs. 7 and 8).

In sections still further into the fusion area, the apical ends of the neuroepithelial cells gradually changed position from facing the gap between the two oppos-

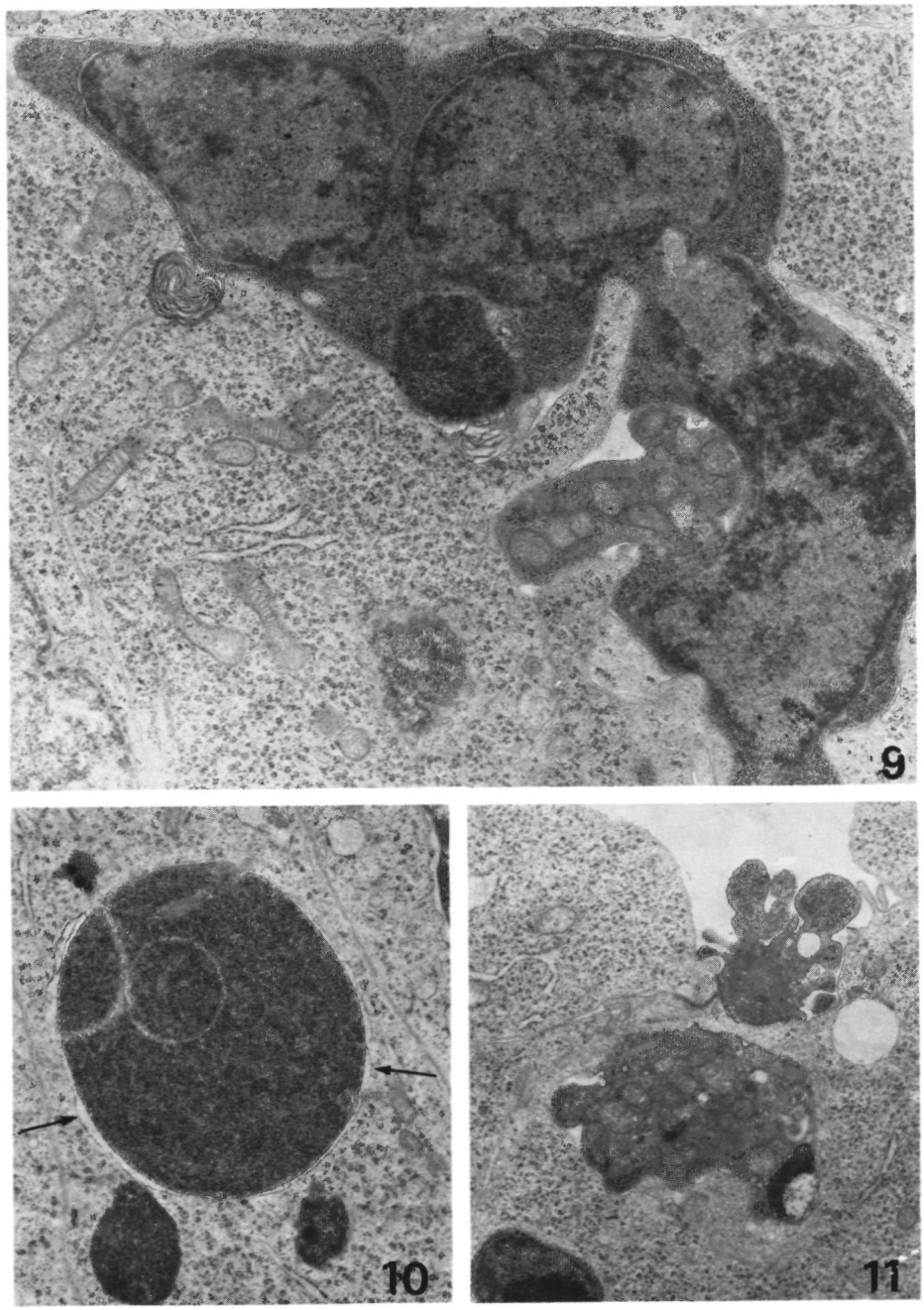


Fig. 9. Degenerating cell characterized by condensation of the nucleus and cytoplasm, the typical features of physiological cell degeneration. Note that a cytoplasmic arm of a healthy neuroepithelial cell begins to penetrate the dying cell. 12,500×

Fig. 10. Degenerating particle phagocytosed by healthy neighbouring neuroepithelial cell. Note the membranes around the phagocytosed particle. 8,000×

Fig. 11. Phagocytosed particles being extruded by the cell into the lumen. Some of the particles contain nuclear, others mainly cytoplasmic remnants. 6,400×

ing walls to facing the lumen of the newly formed tube The impression was gained that the cells rotated about 90 degrees and that the apical ends from opposing cells were sliding along each other till they faced the lumen of the tube Then a typical pseudostratified neuroepithelial pattern was established in the fusion area The surface ectoderm covered the gap only after the neuroepithelial cells had established a pseudostratified epithelium

Closure of the Prosencephalon In this area surface ectoderm and neuroepithelial cells contacted each other approximately at the same time Many thin cell processes extended from the surface ectoderm cells and established distinct junctional complexes similar to those seen in the mesencephalon (Fig 6) The neuroepithelial cells made contact by multiple irregular processes originating from the apical aspects of the cells, but failed to form any membrane specializations Similarly as in the region of the anterior neuropore the neuroepithelial cells soon after fusion formed a regular pseudostratified epithelium with the apical ends at the lumen of the tube where they were connected by junctional specializations

Cell Degeneration. Cell death was observed in the rhombencephalic region before fusion occurred, but usually little cell debris remained when contact was established In the anterior neuropore and prosencephalic regions, however, cell degeneration was seen before, during and after fusion (Fig 7). Only a few cells showed condensation of the nuclear chromatin and cytoplasm, the typical characteristics of on-going physiological cell degeneration (Fig 9) In many cases the degeneration had proceeded to fragmentation and many fragments were surrounded by cytoplasmic arms or completely enclosed by healthy surrounding neuroepithelial cells (Fig 10) In some of the phagocytosed particles the original nuclear components and cytoplasmic organelles could easily be distinguished, but in others those features were lost and only amorphous material could be seen With further degradation clear areas appeared in the phagocytosed cell debris (Fig 7). Initially the phagocytosed particles were surrounded by their own membrane (Fig 10), but later this membrane disappeared (Fig 7) Not infrequently parts of dying cells were extruded into the lumen (Fig 11). Specialized macrophages were not observed Hence, during the fusion process few dying cells were observed, but in the region of the anterior neuropore and prosencephalon the neuroepithelial cells contained many darkly stained particles representing the remnants of phagocytosed degenerated cells The neuroepithelial cells participating in the actual fusion process did not show signs of degeneration themselves

Discussion

In recent years a number of ultrastructural studies have been performed on the fusion of opposing swellings and ridges in the embryo In our own department the fusion of the medial and lateral nasal swellings and of the neural walls was examined with the ruthenium red technique and an opaque substance

was found over the free surface of the epithelial linings (Gaare and Langman, 1977, Sadler, 1978) This surface coat, probably glycoprotein in nature (Leblond and Bennett, 1974, Luft, 1976), was often thickest in the region of prospective contact and tapered off in nasal and oral directions It was suggested that the coat may play an important role in cell recognition and in mediating the initial contact between processes from opposing cells Greene and Kochhar (1974), Souchon (1975), Pratt and Greene (1975), and Pratt et al (1975) similarly found a cell coat over the medial edges of the palatal shelves, the prospective contact regions in the palate When Pratt et al (1975) prevented the formation of the surface coat by treatment with diazo-oxo-norleucine (DON), the palatal shelves failed to adhere, indicating that the coat may be important in mediating adhesion between opposing palatal shelves Examining the fusion of the neural walls Moran and Rice (1975) and Sadler (1978) also found a heavy surface coat on the rims of the neural folds in amphibia and mice, respectively In the mouse embryo the coat was particularly heavy in the region of prospective contact Hence, although in our ultrastructural studies we did not stain for the surface coat, it must be realized that in addition to the direct cellular contact between the cells, the surface coat may also be essential in the closure of the neural tube

Permanent contact between cells of opposing nasal swellings was established by small projections arising from the surface of the cells (Gaare and Langman, 1977) Where the membranes touched, they were temporarily characterized by increased electron density, but distinct cell membrane specializations such as desmosomes were never observed Fusion between the endocardial cushions of the chick heart was studied by Hay and Low (1972) and Los and van Eijndthoven (1973) The initial contact between the cushions was established by small and sometimes tongue-like processes Specialized junctional complexes were never found with exception of a few small junctions in the region of apposition In the contact region between the palatal shelves, Hayward (1969) found a few desmosomes between the contacting epithelial cells, but Farbman (1968) was unable to detect any membrane specializations between the shelves Similarly Hinrichsen and Stevens (1974) failed to see any distinct membrane specializations between the shelves Hence, few if any specialized junctional complexes are found in the fusion zone of the palatal shelves, nasal swellings, and endocardial cushions

The fusion mechanism between the neural walls is different from that in the palate and lip This is not surprising since the epithelial seam formed by the epithelial linings of the palatal shelves and nasal swellings disappears shortly after fusion has been established Similarly the cells lining the endocardial cushions disappear shortly after contact has been established and are transformed into mesenchyme cells (Hay and Low, 1972) When the walls of the neural groove fuse, however, no epithelial or cellular seam is formed and little cell degeneration is seen, with exception of the anterior neuropore and prosencephalic regions An additional difference between the fusion of the palatal shelves, nasal swellings, endocardial cushions, and the neural walls is the presence of electron dense membrane specializations between the surface ectoderm cells This is understandable since the surface ectoderm forms a permanent bridge

between the two sides. This bridge will not disappear in contrast to the epithelial seam between the palatal shelves and nasal swellings.

Contact between the neuroepithelial cells of opposing walls in the prosencephalic and mesencephalic regions is initially established by numerous finger-like cellular projections which interdigitate with each other. These projections, however, fail to form any specialized junctional complexes. Since shortly after fusion, the neuroepithelial cells orientate themselves with their apical ends to the lumen, the formation of specialized junctional complexes would make this orientation very difficult. In all probability the cells "slide" along each other until the final position and orientation have been reached. It seems to us that the closing mechanism in the prosencephalon, the anterior neuropore region and the mesencephalon is principally the same, with the only difference that in the mesencephalic region the surface ectoderm cells make the first contact, while in the other two areas the initial contact is established by the neuroepithelial cells.

The fusion of the rhombencephalon was characterized by some special features. Initially the rhombencephalic wall was made up of surface ectoderm cells with numerous microvilli, neuroepithelial cells with apical protrusions, and a row of flat cells as a transition between the two. At the junction between the flat epithelial cell and the surface ectoderm cell were frequently seen large cytoplasmic extensions protruding into the gap between the two walls. These surface structures were more elaborate than those on the surface ectoderm or at the neuroepithelium and are probably comparable to the ruffles described and illustrated by Waterman (1976). When this investigator studied the closure of the neural groove with the scanning electron microscope, numerous lamellapodia or ruffles were observed to protrude between the presumptive surface ectoderm and the flat cells. It was frequently impossible to determine from which cell type they originated. Indeed, in our own study we sometimes gained the impression that the protrusions originated from the surface ectoderm cells and sometimes from the flat cells.

The contact between the two walls was not only made by the large ruffles but also by extensions from other cells. The nature of this latter type of cells was difficult to determine by morphological characteristics. They did not resemble surface ectoderm cells characterized by microvilli, nor neuroepithelial cells characterized by apical protrusions and sharply delineated tight junctions. In addition, the flat cells seen earlier could not be detected when actual fusion occurred. According to Waterman (1976) the flattened cells participate in the attachment between the neural folds and then lose their extensive contacts with both the surface and neuroepithelial cells to become neural crest cells. This suggestion raises the possibility that the initial contact in the rhombencephalon is established by neural crest cells. Indeed, Martin-Padilla (1970) also suggests that the neural folds in the hamster and mouse make their initial contact at the level of neural crest cells. Apparently the neural crest cells have the ability to form large cytoplasmic arms designed to establish points of adhesion with opposing similar cells (Harris, 1973; Revel, 1974). The neural crest cells of the opposing walls fail to form highly specialized cell junctions. This is understandable since they will migrate bilaterally to participate in the formation

of their respective tissues as soon as firm contact between the surface ectoderm cells has been established Hence, it is highly likely that in the rhombencephalic area both the surface ectoderm and neural crest cells play an important role in the fusion between the walls

Acknowledgments We wish to express our thanks to Miss Linda Chia and Mrs Lynn Ellis for helping us with the technical procedures and Mrs Barbara Stevn for typing the manuscript

This work was supported by grants NS 06188 and DE 07037 to Dr Langman and by an IBRO Fellowship Neurobiology from UNESCO and the Government of The Netherlands to Dr Geelen

References

Bancroft M Bellairs R Differentiation of the neural plate and neural tube in the young chick embryo Anat Embryol **147** 309 335 (1975)

Barson A J Portch P A Scanning electron microscopy of dysraphic human and chick spinal cord Dev Med and Child Neurol **16** (Suppl 32) 152 (1974)

Christie G A Developmental stages in somite and post-somite rat embryos based on external appearance and including some features of the macroscopic development of the oral cavity J Morphol **114** 263 286 (1964)

Edwards J A The external development of the rabbit and rat embryo In Advances in teratology Vol 3 (D H M Woollam ed) pp 239 263 New York Academic Press 1968

Farbman A I Electron microscope study of palate fusion in mouse embryos Develop Biol **18** 93 116 (1968)

Gaare J D Langman J Fusion of nasal swellings in the mouse embryo Surface coat and initial contact Am J Anat **150** 461 476 (1977)

Geelen J A G Langman J Closure of the neural tube in the cephalic region of the mouse embryo Anat Rec **189** 625 640 (1977)

Gouda I G Closure of the neural tube in relation to the developing somites in the chick embryo (Gallus gallus domesticus) J Anat **118** 360 361 (1974)

Greene R M Kochhar D M Surface coat on the epithelium of developing palatine shelves in the mouse as revealed by electron microscopy J Embryol Exp Morph **31** 683 692 (1974)

Harris A K Cell surface movements related to cell locomotion Ciba Foundation Symposia, Elsevier Amsterdam N S **14** 3 26 (1973)

Hay D A Low F L The fusion of dorsal and ventral endocardial cushions in the embryonic chick heart a study in fine structure Am J Anat **133** 1 23 (1972)

Hayward A F Ultrastructural changes in the epithelium during fusion of the palatal processes in rats Arch Oral Biol **14** 661 678 (1969)

Hinrichsen C F L Stevens G S Epithelial morphology during closure of the secondary palate in the rat Arch Oral Biol **19** 969 980 (1974)

Karnovsky M J A formaldehyde glutaraldehyde fixative of high osmolarity for use in electron microscopy J Cell Biol **27** 137A 138A (1965)

Keyser A The development of the diencephalon of the Chinese Hamster Acta Anat (Basel) Suppl 59 (1972)

Leblond C P Bennett G Elaboration of cell coat glycoprotein In The cell surface in development (A A Moscona ed) pp 29 50 New York John Wiley and Sons 1974

Lofberg J Fusion of neural folds and early migration of neural crest cells A scanning and transmission study of the amphibian embryo Doct thesis Uppsala (1974)

Los J A van Eijndhoven E The fusion of the endocardial cushions in the heart of the chick embryo Z Anat Entwickl Gesch **141** 55 75 (1973)

Luft J H The structure and properties of the cell surface coat Int Rev Cytol **45** 291 382 (1976)

Marin-Padilla M The closure of the neural tube in the Golden Hamster Teratology **3**, 39 46 (1970)

Moran D Rice R W An ultrastructural examination of the role of cell membrane surface coat material during neurulation J Cell Biol **62** 172 181 (1975)

Portch P A Barson A J Scanning electron microscopy of neurulation in the chick J Anat **117** 341 350 (1974)

Pratt R M Greene R M The effects of Diazo-oxo norleucine (DON) on development of the palatal epithelium in vitro In New approaches to the evaluation of abnormal embryonic development (D Neubert and H-J Merker eds) pp 648 658 Stuttgart Georg Thieme Publishers 1975

Pratt R M Greene R M Hassell J R, and Greenberg J H Epithelial cell differentiation during secondary palate development In Extracellular matrix influences on gene expression (H C Slavkin and R C Greulich eds) pp 561 565 New York Academic Press 1975

Revel J P Scanning electron microscope studies of cell surface morphology and labeling in situ and in vitro In Scanning electron microscopy/1974 (O Johari and I Corvin eds) pp 541 548 Chicago Illinois Ill Inst Technol Res Inst

Sadler T W Distribution of surface coat material on fusing neural folds of mouse embryos during neurulation Anat Rec in press (1978)

Schluter, G Ultrastructural observations on cell necrosis during formation of the neural tube in mouse embryos Z Anat Entwickl-Gesch **141** 251 264 (1973)

Schroeder T E Neurulation in *Xenopus laevis* An analysis and model based upon light and electron microscopy J Embryol Exp Morphol **23** 427 462 (1970)

Schenefelt R E Morphogenesis of malformations in hamsters caused by retinoic acid relation to dose and stage at treatment Teratology **5** 103–118 (1972)

Souchon R Surface coat of the palatal shelf epithelium during palatogenesis in mouse embryos Anat Embryol **147** 133 142 (1975)

Tarin D Scanning electron microscopical studies of the embryonic surface during gastrulation and neurulation in *Xenopus laevis* J Anat **109**, 535 547 (1971)

Waterman R E SEM observations of surface alterations associated with neural tube closure in the mouse and hamster Anat Rec **183** 95 98 (1975a)

Waterman R E Scanning electron microscopic observations of neural tube closure in the embryonic mouse and hamster Anat Rec **181** 506 (1975b)

Waterman R E Topographic changes along the neural fold associated with neurulation in the hamster and mouse Am J Anat **146** 151 172 (1976)

Accepted January 31 1979

Vitamin A-Induced Anomalies in Young Rat Embryos

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Received for publication April 18, 1973

INTRODUCTION

The earliest manifestations of maternal treatment with excess vitamin A in embryos of golden hamsters were mesodermal alterations and early stages of encephaloschisis (Marin-Padilla and Ferm, 1965 and Marin-Padilla, 1966). The morphogenesis of encephaloschisis after excess vitamin A was studied in mouse embryos starting from the 11th day (Giroud and Martinet, 1960), and in rat embryos from the 11th (Giroud and Martinet, 1957, Giroud, Delmas and Martinet, 1959, Baba and Araki, 1959), and 12th days (Langman and Welch, 1966). In the present study we report the effect of excess vitamin A in young rat embryos. We wanted to find out whether histological alterations can be observed in decidua, giant cells, Reichert's membrane, ectoplacental cone, and yolk-sac placenta, since vitamin A is localized in these structures in hypervitaminotic pregnant rats (Geelen, 1972).

MATERIAL AND METHODS

Female (2-3 month old virgin) and male Wistar rats (T.N.O. Holland) were put together from 6 PM to 8 AM. The morning vaginal smears were examined for sperm. The day on which sperm was found was called the 1st day of pregnancy. Pregnant females were placed in plastic cages and given food and water ad libitum. At approximately 10 AM on specified days of gestation 50,000 IU vitamin A (fat-soluble vitamin A palmitate, Arovit, Roche) was administered by oral intubation (table 1). At certain times afterward the females were killed by exsanguination under ether anesthesia, and the uterus was removed and fixed in Bouin's fluid. Implantation sites were separately embedded in paraffin, and 8μ sections were made in three different planes. In all cases the embryo had a constant position in relation to the uterus. Thus serial sections in transverse, horizontal, and sagittal planes could be produced without manipulation of the delicate embryo. The sections were stained with HE. The experimental specimens were compared with the same number of control embryos.

Table 1 Data about the embryos studied

Group	No of females	Days of treatment	Time of sacrifice	No of implantation sites studied
I	1	7, 8, 9th	10th at 9 AM	2
II	2	7, 8, 9, 10th	10th at 9 PM	5
III	2	7, 8, 9, 10th	11th at 2 AM	5
IV	1	7, 8, 9, 10th	11th at 9 AM	6

RESULTS

10th day, 9 AM

Control No somites had been formed yet The neural groove was shallow and short The rudiment of the allantois had developed

Experimental Both embryos were slightly retarded compared with the controls No anomalies were observed in the embryos, decidua, yolk-sac placenta, ectoplacental cone, giant cells, or Reichert's membrane

10th day, 9 PM

Control One to three pairs of somites had developed The neural groove had become deeper and longer The rostral part of the neuro-epithelium was bulging dorsally above the cephalic mesenchyme The foregut invagination was developing The primitive heart tubes were situated laterally to this structure, continuing rostrally as the dorsal aortae Sinusoids could be observed under the cephalic neuro-epithelium.

Experimental All studied embryos were at the same developmental stage as those of the control group The part of the cephalic mesenchyme, situated immediately under the neural groove, had a reduced number of cells (fig 1) No excessive necrosis or other abnormalities were found in the decidua, giant cells, Reichert's membrane, yolk-sac placenta, ectoplacental cone, or embryos

11th day, 2 AM

Control Five to six pairs of somites were present A small portion of the neural tube was closed The allantois had reached the ectoplacental cone The primitive heart tubes had met in the mid-line, the dorsal aorta and first visceral artery were present, they contained some erythroblasts The median part of the foregut had a small recess extending to the ventral tip of the neural groove A necrotic area (small, round, darkly staining bodies) surrounded the most rostral part of the foregut and head process (fig 2)

Experimental There was slight retardation in comparison with the controls the neural tube was not closed, and the allantois had not reached the ectoplacental

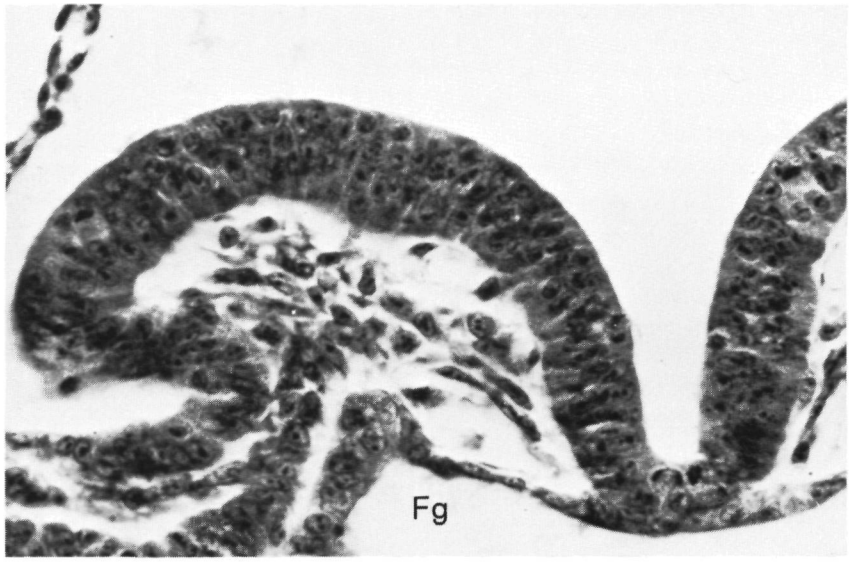

Fig. 1. Transverse section of the cephalic part of a group II experimental embryo. The mesenchyme is insufficient. Fg.: foregut. 420x.

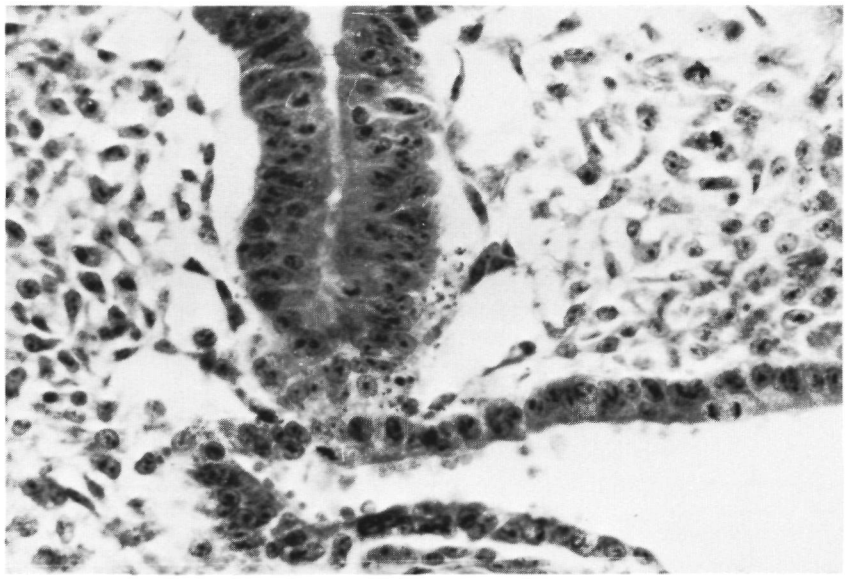

Fig. 2. Transverse section of a group III control embryo. Necrotic cells and darkly staining bodies surround the rostral end of the foregut and head process. The mesenchyme is abundant; the blood vessels are small. 420x.

cone. The blood vessels of the cephalic region were dilated, and the number of mesenchymal cells was reduced in four embryos. Indentations were observed in the basal aspect of the cephalic neuro-epithelium, dorsal to the malformed blood vessels (fig. 3). No anomalies were observed in the decidua, giant cells, Reichert s membrane, yolk-sac placenta, and ectoplacental cone.

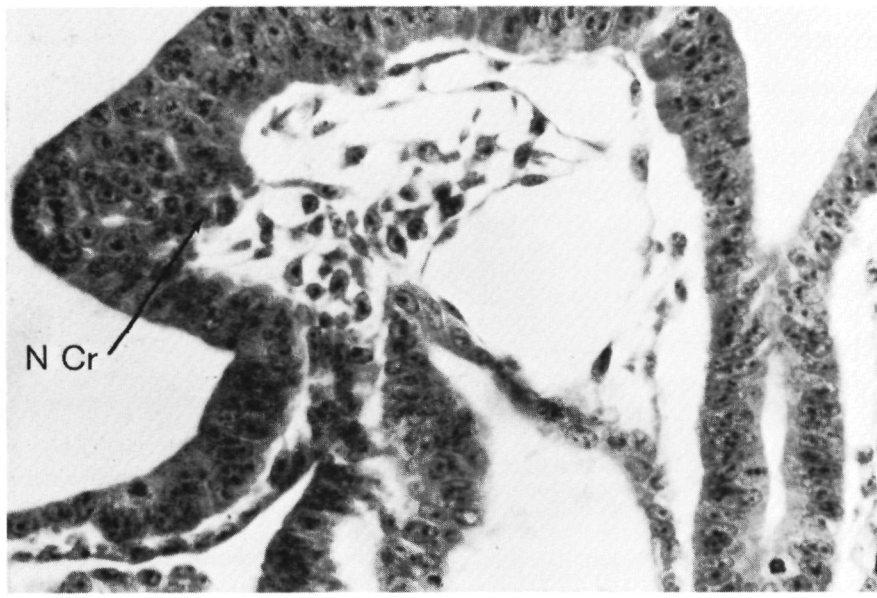

Fig. 3. Transverse section of the cephalic part of a group III experimental embryo. The blood vessels are dilated; the ventral aspect of the neuro-epithelium is malformed. Compare with control embryo of fig. 2. N.Cr.: neural crest cells. 420x.

11th day, 9 AM

Control: The 2 AM control group of the 11th day was at the same developmental stage as the 9 AM experimental group of the 11th day.

Experimental: The dilation of the cephalic vessels was increased (figs. 4, 5). The dorsal recess of the foregut was small, and it was situated beside the ventral tip of the neural groove in some embryos. In one embryo an extensive necrotic area was observed in the primitive streak. In two specimens many pycnotic cells were found in the fold connecting parietal and visceral yolk-sac epithelia. A large necrotic area was observed in the central part of the decidua basalis of another specimen. In one specimen a large blood-filled bleb was found on the decidual mass, protruding into the uterine cavity. No abnormalities were seen in the decidua capsularis, giant cells, Reichert's membrane, and ectoplacental cone.

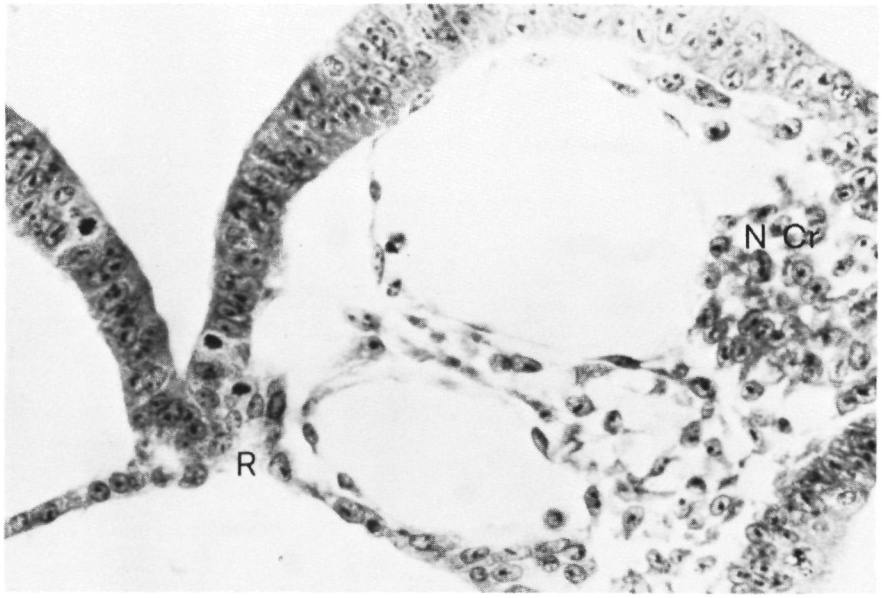

Fig. 4. Transverse section of the cephalic part of a group IV experimental embryo. The dorsal recess (R) of the foregut lies laterally to the ventral tip of the neural groove. N.Cr.: neural crest cells. 420x.

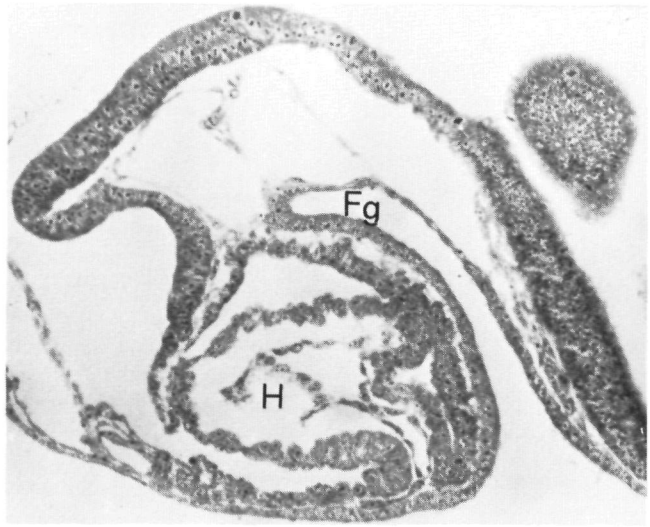

Fig. 5. Paramedian section of the head region of a group IV experimental embryo. H.: heart; Fg.: foregut. 160x.

DISCUSSION

In the experimental embryos there was a decreased number of cephalic mesenchymal cells, and greatly expanded rostral blood vessels in groups II, III, and IV. Since the number of pycnotic cells found in the experimental embryos was normal, we suppose that reduced mitotic activity accounts for this mesenchymal underdevelopment, and that the latter phenomenon is followed by vascular dilation, so that finally most of the subneural area becomes occupied by embryonic blood vessels.

This process takes place during the transformation of the densely packed mesodermal cells into the more loosely arranged mesenchymal cells. Apparently this metamorphosis is especially sensitive to the teratogenic effect of vitamin A.

In a previous experiment, after maternal treatment with 50,000 IU vitamin A palmitate on the 8th, 9th, and 10th days of gestation, we obtained two 13th-day rat fetuses with caudal myeloschisis, dilation of the dorsal aortae, and almost complete absence of mesenchymal cells (fig. 6). This caudal anomaly shows a striking resemblance to the rostral malformations observed in younger stages. Similar cephalic anomalies were found by Bonnevie (1940) in mice with hereditary exencephaly.

Marin-Padilla and Ferm (1965) found necrosis of rostral somites in golden hamster embryos 12 h after maternal administration of 20,000 IU vitamin A; after 24 h they found massive necrosis of somites and focal necrosis in the notochord. However, in our material the number of necrotic cells in the somites was similar in control and experimental embryos, and notochordal necrosis was found only rostrally in controls. The dilation of vascular spaces observed by Marin-Padilla (1966) is also present in our embryos.

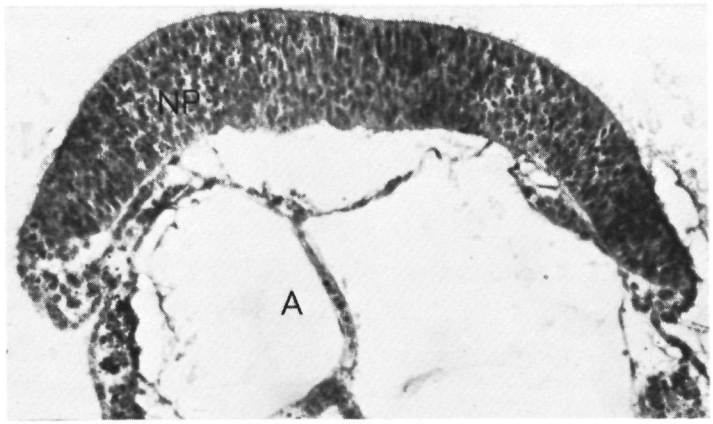

Fig. 6. Transverse section of vitamin A induced myeloschisis in a 13th-day rat fetus. The dorsal aortae (A) are greatly dilated; only a few mesenchymal cells can be observed. N.P.: neural plate. 165x.

A preliminary investigation showed that administration of 50,000 IU vitamin A orally on each of the 7th, 8th, 9th, and 10th day of gestation produced over 75% exencephalic 12th-and 13th-day fetuses, but approximately 100% resorption on the 14th, 15th, and 16th days Thus the procedure employed in the present experiment is very appropriate for producing a great percentage of young exencephalic embryos, and it can be used for the study of the early development of exencephaly

It seems to us that the cephalic mesenchymal anomaly is an important factor in the morphogenesis of schisencephaly Three hypothetical mechanisms may be involved (1) There may be insufficient induction of neurulation, caused by the hypoplasia of the mesodermal elements (2) Owing to the mesenchymal derangement and collapse the neural plate is flattened, consequently the lateral parts of the neuro-ectoderm cannot reach the dorsal mid-line where neural closure has to take place In the group IV experimental embryos the mesenchymal collapse is demonstrated by the fact that the ventral tip of the neuro-ectoderm is situated laterally to the dorsal recess of the roof of the foregut (3) The abnormal arrangement of the neuro-ectoderm might be involved in the development of schisencephaly For Jelinek and Friebova (1966) showed that the neuro-ectodermal proliferation in a transverse plane results in neurulation since the neuro-ectodermal cells are firmly anchored to the internal limiting membrane A normal cellular arrangement is necessary for this mechanism In our group III experimental embryos cellular derangement is clearly visible in the ventral part of the neuro-epithelium It still remains questionable whether this derangement is influenced by the anomalies of the underlying blood vessels

Necrotic areas were observed in two group IV experimental specimens in structures (decidua basalis and the fold connecting parietal and visceral yolk-sac epithalia) in which vitamin A accumulation has been found (Geelen, 1972) Although this phenomenon was found in only two specimens, it suggests that excess vitamin A has a cytotoxic effect on the extraembryonic tissues However, more detailed experiments will be necessary to support our opinion (Geelen, 1972) that a deleterious influence on the decidua and yolk-sac epithelium is a factor in vitamin A teratogenesis

SUMMARY

The early conceptuses of Wistar rats, treated orally with 50,000 IU vitamin A palmitate on each of the 7th, 8th, 9th, and 10th or 7th, 8th, and 9th day of pregnancy, were studied In the young somite stages shortage of cephalic subneural mesenchyme, dilation of the rostral bloodvessels and derangement of the cephalic neuro-epithelium were found The relationships between the anomalies and the genesis of schisencephaly is discussed

Key-words vitamin A – anomalies – embryos – rat

REFERENCES

BABA, T and I ARAKI Morphogenesis of malformation due to excessive vitamin A I Morphogenesis of exencephaly Osaka City Med J 5 (1959) 9-15

BONNEVIE, K Handbuch der Erbbiologie des Menschen Vol I G Just, ed Springer, Berlin (1940)

GEELEN, J A G The localization of vitamin A in the pregnant rat by means of fluorescence microscopy Teratology 6 (1972) 19 26

GIROUD, A, A DELMAS and M MARTINET Etude morphogénétique sur des embryons anencéphales Arch Anat 42 (1959) 203-230

GIROUD, A and M MARTINET Morphogénese de l'anencéphalie Arch Anat Micr Morph Exp 46 (1957) 247-264

Anencéphalie expérimentale chez la souris et comparaisons avec l'anencéphalie chez l'homme Acta Anat 43 (1960) 358 370

JÍLINEK, R and Z ERBOVA Influence of mitotic activity on neurulation movements Nature 209 (1966) 822 823

LANGMAN, J and G WELCH Effect of vitamin A on development of the central nervous system J Comp Neur 128 (1966) 1-16

MARIN-PADILLA, M. Mesodermal alterations induced by hypervitaminosis A J Embryol Exp Morphol 15 (1966) 261 269

MARIN-PADILLA, M and V H HERM Somite necrosis and developmental malformations induced by vitamin A in the golden hamster J Embryol Exp Morphol 13 (1965) 1-8

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Anat Embryol 159, 223–234 (1980)

Anatomy and Embryology

The Influence of Excess Vitamin A on Neural Tube Closure in the Mouse Embryo

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Summary. The effect of excess vitamin A on the closure of the neural tube in mouse embryos was examined with light microscopy, transmission and scanning electronmicroscopy. The embryos were treated with the vitamin just before closure of the brain vesicles and examined during the following 24 h, a period during which under normal conditions the brain completely closes.

At 18–24 h after treatment the external features of the treated specimens began to differ from those of the controls. In the treated embryos the neural walls folded laterally and became widely separated, whereas those of the controls folded dorsomedially and fused in the midline. Histologically, the first difference between treated and control embryos was noted at two hours after treatment, when large intercellular spaces appeared between the neuroepithelial cells of the treated embryos. These spaces were mainly present between the apical ends of the wedge-shaped neuroepithelial cells. This accumulation of intercellular spaces interfered with the normal morphogenetic movement of the neural walls, which remained convex instead of becoming concave. This convex bending resulted in non-closure of the neural tube.

In addition to the appearance of large intercellular spaces some neuroepithelial cells as well as some mesenchymal, endothelial, and surface ectoderm cells showed swelling and degeneration as a result of the vitamin A treatment. This cell degeneration probably contributes to failure of the neural tube to close due to loss of cohesion at the luminal surface and the lack of mesenchymal support needed for the elevation of the neural walls. However, the increase of intercellular spaces at the apical side of the neuroepithelium is in all probability the major cause for the failure of the neural tube to close.

Key words: Vitamin A – Neural tube closure – Exencephaly – Anencephaly.

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Introduction

Neural tube formation in the mammalian embryo begins with the appearance of a groove in the primitive neural plate As the groove gradually deepens the tips of the opposing neural walls approach each other in the dorsal midline, establish contact and finally fuse The transformation of the neural plate into the neural groove and subsequently into the neural tube is thought to result from the interaction of the cellular and tissue characteristics of the neuroepithelium The neuroepithelial cells have a wedge-shape with a narrow apical end and a much wider basal part This shape is maintained by a circular band of microfilaments at the apical end and by microtubules oriented along the long axis of the cell (Karfunkel, 1973) In addition, the cells are tightly connected to each other by junctions at the luminal surface (Jelinek and Friebova, 1966) During the intermitotic phase the wedge-shaped cells extend from the lumen to the basement membrane, but during mitosis they contract to the lumen and become temporarily round After cell division is completed the cells assume their typical wedge-shape and the nuclei migrate again to the periphery (Langman et al , 1966) The wedge-shape of the cells their intercellular connections at the apical ends and the interkinetic migration are thought to play an important morphogenetic role in the formation of the neural tube

Maternal administration of excess vitamin A prevents the closure of the cephalic part of the neural tube and causes the development of exencephaly and subsequently anencephaly in a high percentage of fetuses (Giroud, 1960, Kalter, 1968, Lemire et al , 1978, Geelen 1979) Hence the aim of this work was to examine the effect of excess vitamin A on the shape of the individual neuroepithelial cell and on the contours of the neuroepithelium of the neural wall in mouse embryos during closure of the tube

After the neural groove has been formed and the tips of the neural walls have approached each other, the actual fusion process takes place In the mouse fusion begins in the cervical region, continues to the rhombencephalon and at about the same time occurs in the prosencephalon (Waterman, 1976 Geelen and Langman, 1977, 1979) At the ultrastructural level the opposing neural walls establish contact by interdigitating cell processes This is followed by the formation of intercellular junctions between neuroepithelial and surface ectoderm cells of the opposing neural walls Thus, after the tips of the walls have come close together as a result of the morphogenetic forces of the neuroepithelium, the final closure is accomplished by a fusion process Therefore the second aim of this study was to determine whether vitamin A induced failure of closure is caused by insufficient approximation of the neural walls or by a defect in the actual fusion process

Materials and Methods

Female Swiss mice or ICR mice were mated and examined for the presence of vaginal plugs The day on which the plug was found was considered as day 1 of gestation On day 9 at approximately 9 A M either 10 000 IU vitamin A palmitate or 20 000 IU vitamin A alcohol were administered

by oral intubation. The 9 A.M. time was chosen since at this time in most embryos the formation of the neural groove in the cephalic region has just started and in the cervical region fusion of the neural walls is about to begin (Theiler 1972, Geelen and Langman, 1977). As closure of the primitive brain in the mouse is accomplished in 24 h, the control and treated females were sacrificed at 2, 4, 8, 12, 18 and 24 h after 9 A.M. of day 9. The uterus was removed under ether anesthesia, subsequently the embryos were taken out of the uterine horns and fixed in modified Karnovsky solution (1965). After postfixation in 1% OsO_4 and dehydration in ethanol the tissue was embedded in Araldite. Embryos of the appropriate developmental stage were then selected, properly oriented, cut at 1 µm with a Sorval, Porter-Blum MT$_2$ ultramicrotome and stained with toluidine blue. In some embryos specific areas were cut for electron microscopy. These sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100S electron microscope.

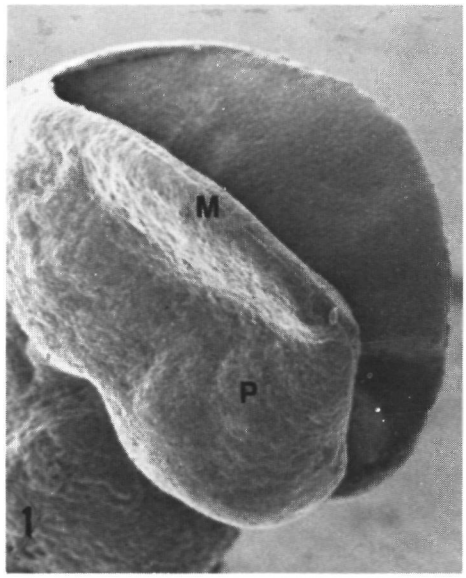
The embryos which were selected for scanning electron microscopy were critical point dried after fixation and dehydration and sputter coated with 100 Å gold alloy. They were subsequently examined with a JEOL JSM-35C scanning electron microscope.

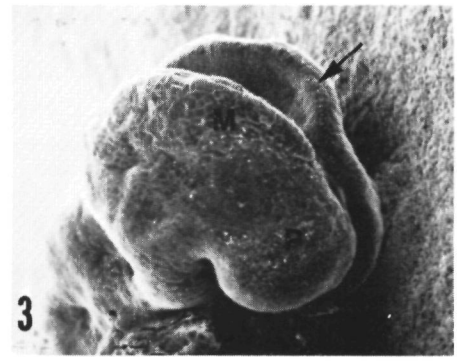
Results

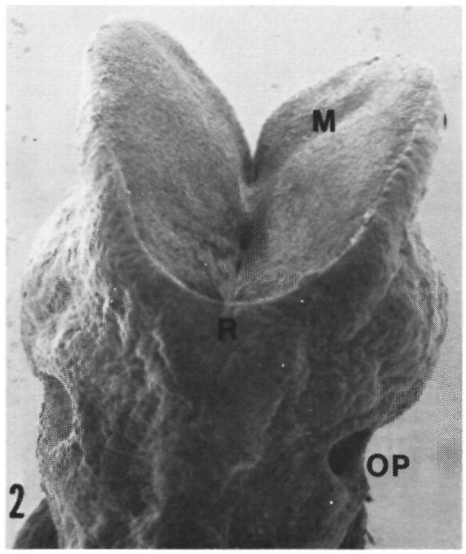
When two hours after treatment with vitamin A the external features of the embryos (2–6 somites) were examined, it was found that in the most advanced specimens the neural tube was closing in the cervical region, while in the cephalic area groove formation had just begun. At 4 and 8 h after treatment (5–12 somites) fusion of the neural walls had proceeded to the rhombencephalon. Twelve hours after treatment (10–15 somites) fusion in the rhombencephalon was proceeding rostrally; in the prosencephalon and mesencephalon the neural walls were approaching each other, but in some specimens they were still folded laterally. When treated embryos were compared with controls it was impossible to detect any difference between the two groups in the overall shape and contours of the neural walls up to 12 h after treatment.

Eighteen hours after treatment (15–20 somites) the brain vesicles were closed in 7 out of 26 vitamin A treated embryos. In most of the remaining embryos the medial side of the neural walls was concave, the tips were bending toward the dorsal midline and closure was nearly finished. In four embryos, however, the medial side of neural walls was convex and the tips still pointed in a dorsolateral direction, a feature which might be considered as the first manifestation of the development of exencephaly. At 24 h after treatment the brain vesicles of two thirds of the embryos were closed, but the others showed a distinct convexity and lateral bending of the neural walls in the prosencephalon and most conspicuously in the mesencephalon. Hence, a normal embryo could be distinguished from an exencephalic embryo in its external features only at 18–24 h after treatment.

To obtain a more detailed picture scanning electronmicrographs were made of the rostrolateral and posterior aspects of the brain vesicles of abnormal embryos at 24 h after treatment. When these embryos (Figs. 1 and 2) were compared with 9-somite controls (Figs. 3 and 4), it was evident that both in treated and untreated embryos closure of the neural groove had proceeded to about the same level in the rhombencephalon. In the treated embryos the mesencephalic walls were convex, widely separated and pointing in dorsolateral







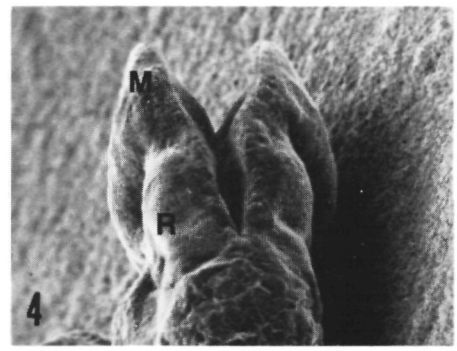


Fig. 1. Scanning electronmicrograph of a 20-somite embryo, 24 h after treatment with vitamin A. Rostrolateral view. Note the wide open mesencephalon and the convexity of the neural walls. *M* mesencephalon; *P* prosencephalon. ×75

Fig. 2. Scanning electronmicrograph of the same embryo as in Fig. 1, but seen from dorsal. Note the wide open mesencephalon and the convexity of the neural walls. *M* mesencephalon. *OP* otic pit. *R* rhombencephalon. ×75

Fig. 3. Scanning electronmicrograph of a 9-somite control embryo. Rostrolateral view. Note that the medial side of the neural walls in the mesencephalon is concave (*arrow*). *M* mesencephalon; *P* prosencephalon. ×75

Fig. 4. Scanning electronmicrograph of a 9-somite control embryo. Dorsal view. Note that the neural walls of the rhombencephalon and mesencephalon are approaching each other and are pointing in dorsal (dorsomedial) direction. *M* mesencephalon; *R* rhombencephalon. ×75

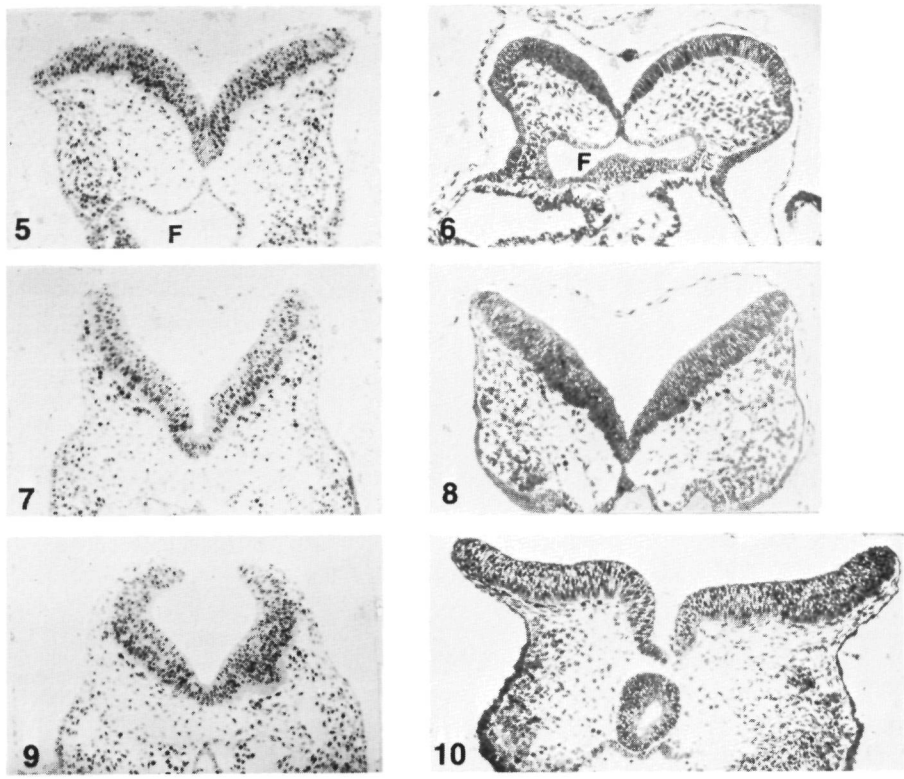


Fig. 5. Transverse section through the cephalic region of a 6-somite control embryo. Note the convexity of the medial sides of the neural walls. *F* foregut. × 75

Fig. 6. Transverse section through the cephalic region of a treated embryo, 2 h after treatment. The neural walls are convex as in the controls. *F* foregut. × 75

Fig. 7. Transverse section through the cephalic region of a 10-somite control embryo. The medial sides of the neural walls begin to assume a concave shape. × 75

Fig. 8. Transverse section through the mesencephalon of a treated embryo, 12 h after treatment. Note that the neural walls have a V-shape and that the medial sides are slightly convex. × 75

Fig. 9. Transverse section through the cephalic part of a 15-somite control embryo. The medial sides of the neural walls are concave and the tips are bent in dorsomedial direction. × 75

Fig. 10. Transverse section through the cephalic part of a treated embryo, 24 h after treatment. Groove formation has occurred in the ventromedial part, but most of the walls are widely separated. × 75

direction (Fig. 2). In the controls the walls of the mesencephalon were already slightly concave and pointing in dorsomedial direction (Fig. 4).

To compare our scanning data with histological studies, serial sections were made through the mesencephalon of control and treated embryos. When two hours after treatment the contours of the neural walls were studied they were found to be convex in both treated and controls (Figs. 5 and 6). During the

next 10 h the neural walls assumed a V-shape and the convexity of the walls decreased (Figs 7 and 8) In some specimens the neural wall was slightly concave, but at this stage of development it was impossible to distinguish between a normal and an exencephalic embryo

During the next few hours the neural walls of the treated embryos failed to bend in dorsomedial direction and continued to grow in dorsolateral direction (Fig 10) In the ventral midline some groove formation occurred, but this was usually minimal In the controls the medial side of walls became concave and the tips bent in dorsomedial direction (Fig 9) Thus, while growth of the neuroepithelium was about normal and the wall doubled in thickness groove and tube formation were disturbed The neural walls remained convex instead of becoming concave as under normal conditions

To obtain more cellular detail about the action of vitamin A on the neuroepithelium the treated embryos were examined with light and electron microscopy In a 4-somite control embryo the neuroepithelium formed a regular pseudostratified layer (Fig 11) In some areas only one nuclear layer could be distinguished but in other areas two or three rows of nuclei were found Most of the nuclei were located in the basal part of the cell and many cells were clearly wedge-shaped with a narrow apical and a wider basal end Dividing cells were located at the prospective lumen and were large, pale and round Occasionally intercellular spaces were noted, but they were narrow, elongated and not conspicuous In 10–20 somite controls the neuroepithelium had considerably increased in thickness and 4 to 5 rows of nuclei were present (Figs 13 and 15) Many mitotic cells were found at the lumen and the luminal surface was characterized by small protruding cytoplasmic blebs (Fig 13) Small round dark particles indicative of cell death were observed in the neuroepithelium, but cell death was not prominent

When treated embryos were examined it was found that at two hours after treatment the cells were separated by large intercellular clefts which were substantially wider and more frequent than those observed in the controls (Fig 12) The intercellular connections at the lumen remained intact At 12 and 24 h after treatment the neuroepithelium had considerably increased in thickness and mitotic cells were visible at the lumen (Figs 14 and 16) The intercellular clefts were still present in large numbers Since they were particularly seen at the apical ends of the cell, the overall contour of the neuroepithelial wall failed to become concave and it is thought that this disturbance is the basic cause of the failure of the neural groove to close

In addition to the appearance of wide intercellular spaces a number of cells was seen to degenerate as soon as 2 h after treatment This process started with loss of density of the cytoplasm and was followed by swelling of the nucleus (Figs 17, 18 and 19) Subsequently the cytoplasm disappeared and only a pale nucleus remained In the final stage the nucleus underwent lysis In some embryos only a few cells were seen to degenerate, but in other embryos large numbers of degenerating cells were located in the neuroepithelium as well as in the mesenchyme, endothelium and surface ectoderm In the most extreme cases the damage was so widespread, that the embryo probably would not survive In the least affected cases cell degeneration affected the neuroepithe-

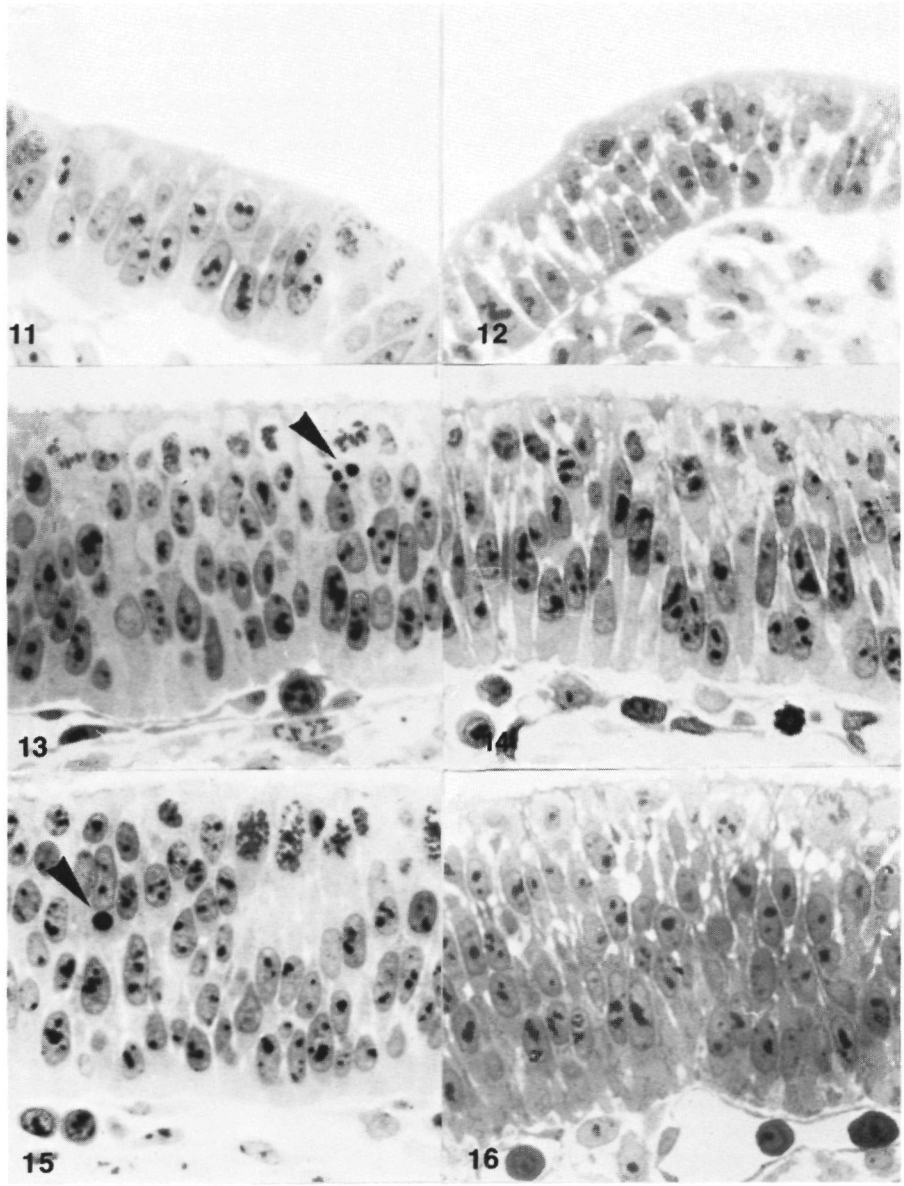


Fig. 11. Neuroepithelium of 9-somite control embryo. ×470

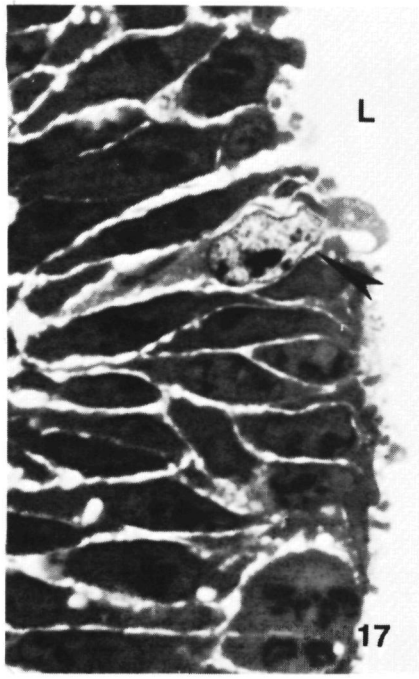
Fig. 12. Neuroepithelium of treated embryo, 2 h after treatment. Note the presence of large intercellular spaces in the neuroepithelium. ×470

Fig. 13. Neuroepithelium of 12-somite control embryo. Several dark particles indicative of physiologic cell death (*arrowhead*) are visible. ×470

Fig. 14. Neuroepithelium 12 h after treatment. Note the presence of intercellular spaces between the apical ends of the cells. ×470

Fig. 15. Neuroepithelium of 20-somite control embryo. Several dark particles indicative of physiologic cell death are visible (*arrowhead*) ×470

Fig. 16. Neuroepithelium of treated embryo, 24 h after treatment. The intercellular spaces are particularly seen in the apical region of the neuroepithelium. ×470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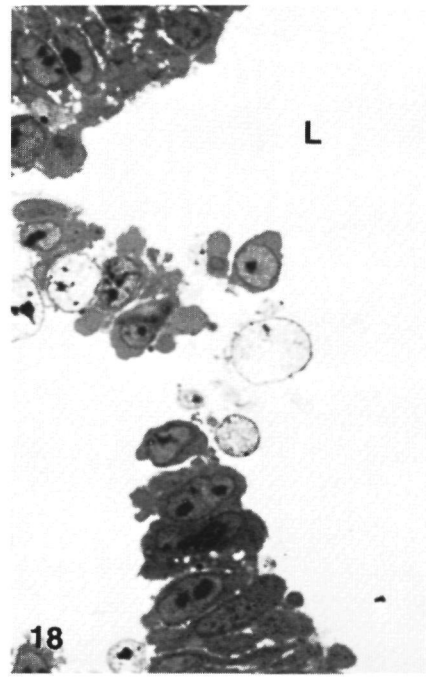
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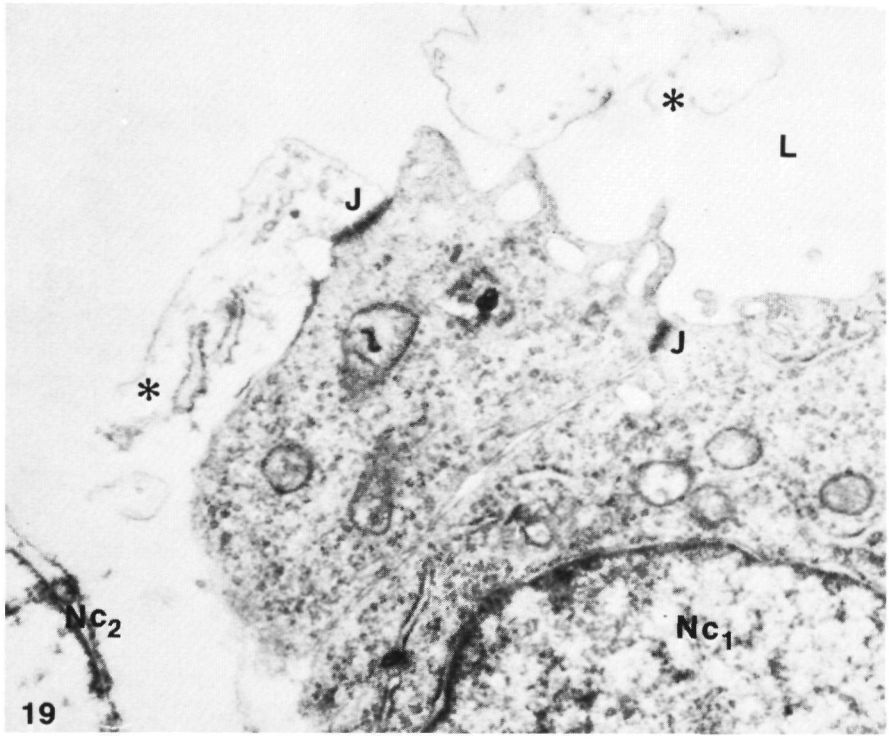
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lium sometimes at the luminal surface and sometimes in the peripheral parts. When a cell at the presumptive luminal surface was affected its remnants were extruded and the continuity of neuroepithelial surface was temporarily disrupted.

Based on our findings it is suggested that the widespread increase of the intercellular spaces in the apical parts of the neuroepithelium abolishes the effect of the wedge-shape of the neuroepithelial cells and is the primary cause for failure of the neural tube to close. This failure to close may be supported by temporary disruption of the luminal surface as a result of cell degeneration. No indication was found to suggest that the vitamin A induced failure of closure was caused by a defect in the actual fusion process; on the contrary all data indicate that non-closure of the neural tube is caused by insufficient approximation of the neural walls.

Discussion

In the present experiments excess vitamin A was administered to pregnant mice and its effect on the invagination of the neural plate and the fusion of the neural walls was investigated. The formation of the neural groove was disturbed, the medial sides of the neural walls remained convex instead of becoming concave and the tips bent in dorsolateral direction instead of in dorsomedial direction.

Several hypotheses have been proposed to explain the insufficient groove formation and the failure of the neural walls to meet in the dorsal midline. Marin-Padilla (1966), Morriss (1973), and Geelen (1972) suggested that failure of the neural walls to elevate and to fuse was due to cell degeneration, increase of intercellular space and dilation of blood vessels in the lateral head mesenchyme. Other investigators suggested that the failure of the neural groove to form and of the neural tube to close due to excess vitamin A was to be found within the neuroepithelium itself (Langman and Welch, 1966; Theodosis and Fraser, 1978). These authors suggested that neurulation is mainly caused by the typical wedge-shape of the neuroepithelial cells, which is maintained by microfilaments, microtubules and intercellular junctions at the lumen (Kar-

Fig. 17. Section through the neuroepithelium of an exencephalic embryo, 24 h after treatment Note the degenerating cell (*arrowhead*) which is characterized by loss of density and vacuolization of the cytoplasm and swelling of the nucleus. Part of the cytoplasm is extruded into the lumen. *L* presumptive lumen × 1,290

Fig. 18. Section through the neuroepithelium of an embryo, 2 h after treatment The neuroepithelium is severely damaged and the continuity is completely lost. Cells in various stages of degeneration are present × 720

Fig. 19. Electronmicrograph of a section through the neuroepithelium of an embryo, 2 h after treatment This picture shows two neuroepithelial cells and the remnants of a cell affected by the vitamin A treatment The cells are connected by intercellular junctions at the lumen Nc_1-nucleus of neuroepithelial cell; Nc_2-nuclear remnant of degenerated cell *L* presumptive lumen, *J* junction. * Cytoplasmic remnants of degenerated cell. × 15,000

funkel, 1973) Interference with any of these structures theoretically should lead to abnormal neurulation

To test this theory Lee and Kalmus (1976) treated chick embryos prior to neural tube closure with cytochalasin B a compound thought to act on the microfilaments that produce interkinetic nuclear migration (Hinds and Ruffet, 1971) and cytokinesis (Carter, 1967) Interkinetic migration was inhibited and mitotic cells were seen distributed throughout the neuroepithelium instead of at the lumen The abnormal position of the mitotic cells changed the characteristic neuroepithelial structure essential for the normal neurulation and neurulation failed to occur Similarly when dividing neuroepithelial cells were blocked in metaphase by vincristine treatment (Langman et al , 1966) interkinetic migration was disturbed and the metaphase cells accumulated at the lumen As a result the neural wall remained convex and the groove failed to close When Karfunkel (1972) treated chick embryos with colchicine a substance that disrupts the cellular microtubules, the elongated neuroepithelial cells rounded up at the lumen Again the neural tube failed to close due to the loss of the wedgeshape of the cells Hence all these experiments suggest strongly that interference with the wedge-shape of the neuroepithelial cells results in failure of the tube to close

Excess vitamin A increases the cell generation time and affects all stages of the cell cycle equally (Langman and Welch 1967) However, since all phases are lengthened in equal proportion, there should be no effect on the distribution of cells in different stages of the cycle within the neuroepithelium Indeed in our vitamin A treated embryos the mitotic cells were located at the lumen but no accumulation took place Hence, excess vitamin A does not seriously affect the interkinetic nuclear migration and its effect on the cell cycle does not interfere with the wedge-shape of the cells

The disruptive effect of excess vitamin A on cells, previously observed in cultured cells (Daniel et al , 1966, Lucy and Dingle, 1964) and in mammalian embryos (Morriss, 1973, Theodosis and Fraser 1978), was also found in our experiments The resulting cell death causes some loss of continuity at the presumptive luminal surface and some disturbance of the characteristic structure of the neuroepithelium However, since cell death did not occur frequently, it is not likely that cell degeneration had any serious effect on closure of the neural tube

The major abnormality seen in the vitamin A treated embryos was the striking increase in intercellular space between the neuroepithelial cells, mainly between the narrow apical parts of the cells Since the close apposition of the apical ends of the neuroepithelial cells is thought to be one of the essential morphogenetic factors in the formation of the neural tube, the increase of intercellular space in the apical region will interfere with neurulation in the same manner as the accumulation of mitotic figures at the lumen

The present morphologic study shows that maternal treatment with excess vitamin A causes enlargement of the intercellular spaces in the neuroepithelium This phenomenon was previously described to occur in all parts of the embryo (Morriss, 1973) and was interpreted as a change in the fluid balance, probably due to the disruptive effect of excessive vitamin A on lipoprotein membranes

The increase of extracellular fluid, however, was so pronounced that it did not seem to be caused solely by a shift of fluid from the embryonic cells into the intercellular spaces. In all probability the high concentration of vitamin A found in the yolk sac placenta of vitamin A treated rats and mice (Geelen, 1972; Kochhar, 1976), interferes with the transport functions of the primitive placenta and thus contributes also to the intercellular fluid accumulation. Hence, it seems not unlikely that high doses of vitamin A administered prior to neural tube closure affect the membranes of the embryonic and yolk sac placenta cells resulting in an increase in intercellular fluid. This increase in cellular fluid combined with vitamin A induced cell degeneration probably affects the cephalic mesenchyme and thus has a deleterious influence on the elevation of the neural walls. However, the increase of intercellular fluid in the neuroepithelium and particularly in the apical region directly interferes with the effect of the wedge-shape of the cells is considered to be a most important morphogenetic factor in the formation of the neural tube.

Acknowledgements The assistance of Dr. Paul Peters and Aart Verhoef of the National Institute of Public Health and of Tiny Jansen-van Kempen of the Department of Neurology, University of Nymegen, The Netherlands, is gratefully acknowledged. The technical assistance of Betsy Cochrane and of Mr. Sid Breese and Dahna Haberman of the Central EM Facility is greatly appreciated. Furthermore, the authors wish to express their gratitude to Mrs. Susan Seiler and Mrs. Mary Staton for typing the manuscript. This work was supported by NIH grants no. NS-06188 and DL 07037 to Dr. Langman.

References

Carter SB (1967) Effects of cytochalasins on mammalian cells. Nature 213, 261–264

Daniel MR, Dingle JT, Glauert AM, Lucy JA (1966) The action of excess vitamin A alcohol on the fine structure of rat dermal fibroblasts. J Cell Biol 30, 465–475

Geelen JAG (1972) The localization of vitamin A in the pregnant rat by means of fluorescence microscopy. Teratology 6, 19–26

Geelen JAG (1972) Vitamin A-induced anomalies in young rat embryos. Acta Morph. Neerl-Scand. 11, 233–240

Geelen JAG (1979) Hypervitaminosis A induced teratogenesis. Critical Reviews in Toxicology. CRC Press, West Palm Beach 6, 351–375

Geelen JAG, Langman J. (1977) Closure of the neural tube in the cephalic region of the mouse embryo. Anat. Rec. 189, 625–640

Geelen JAG, Langman J. (1979) Ultrastructural observations on closure of the neural tube in the mouse. Anat. Embryol. 156, 73–88

Giroud A. (1960) Causes and morphogenesis of anencephaly. In: GE W. Wolstenholme and CM O'Connor (eds). Little Brown and Co., Boston USA. CIBA-foundation symposium in Congenital Malformations

Giroud A, Delmas A, Martinet M. (1959) Etude morphogenetique sur des embryons anencephales. Arch. Anat. Histol. Embryol. 42, 203–230

Hinds JW, Ruffet TL. (1971) Cell proliferation in the neural tube. An electronmicroscopic and Golgi analysis of the mouse cerebral vesicle. Z. Zellforsch. 115, 226–264

Jelinek R, Friebova Z. (1966) Influence of mitotic activity on neurulation movements. Nature 209, 822–823

Kalter H. (1968) Teratology of the central nervous system. Univ. of Chicago Press, Chicago

Karfunkel P. (1972) The activity of microtubules and microfilaments in neurulation in the chick. J. Exp. Zool. 181, 289–302

Karfunkel P. (1973) The mechanism of neural tube formation. Int. Rev. Cytol. 38, 245–271

Karnovsky MJ (1965) A formaldehyde glutaraldehyde fixative of high osmolarity for use in electron-microscopy J Cell Biol 27 137A 138A

Kochhar DM (1976) Transplacental passage of label after administration of ^{3}H-retinoic acid (vitamin A acid) to pregnant mice Teratology 14 53 64

Langman J Welch GW (1967) Excess vitamin A and the development of the cerebral cortex J Comp Neur 131 15 26

Langman J, Guerrant R Freeman B (1966) Behavior of neuroepithelial cells during closure of the neural tube J Comp Neur 127 399 412

Lee HY (1976) Inhibition of neurulation and interkinetic nuclear migration by concanavalin A in explanted early chick embryos Dev Biol 48 392 399

Lee HY Kalmus GW (1976) Effects of cytochalasin B on the morphogenesis of explanted early chick embryos Growth 40 153 162

Lee HY Nagele RG Kalmus GW (1976) Further studies on neural tube defects caused by concanavalin A in early chick embryos Experientia 32 1050–1052

Lemire RJ Beckwith JB Warkany J (1978) Anencephaly Raven Press New York

Lucy JA, Dingle JT (1964) Fat-soluble vitamins and biological membranes Nature 204 156 160

Marin Padilla M (1966) Mesodermal alterations induced by hypervitaminosis A J Embryol Exp Morph 15 261 269

Morriss GM (1973) The ultrastructural effects of excess maternal vitamin A on the primitive streak stage rat embryo J Embryol Exp Morph 30 219 242

Theiler K (1972) The house mouse Development and normal stages from fertilization to four weeks of age Springer Verlag Berlin Heidelberg

Theodosis DT Fraser F (1978) Early changes in the mouse neuroepithelium preceeding exencephaly induced by hypervitaminosis A Teratology 18 219 232

Waterman RF (1976) Topographical changes along the neural fold associated with neurulation in the hamster and mouse Am J Anat 146 151 172

Accepted January 16 1980

Reprinted from TERATOLOGY
Vol 19, No 2, April 1979 © The Wistar Institute Press 1979

Light Microscopic and Ultrastructural Observations in Advanced Stages of Induced Exencephaly and Spinal Bifida

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ABSTRACT This investigation was performed to demonstrate the morphologic basis of the elevation of fetal proteins in the amniotic fluid of fetuses with neural tube defects. Pregnant rats were treated with hypervitaminosis A to induce exencephaly or with trypan blue to produce spina bifida aperta. The malformations were studied on days 15-20.

On day 15 of gestation, edema developed in the primitive nervous tissue. This was followed by the appearance of quickly expanding hemorrhages throughout the ventricular and intermediate zones. Some capillaries did not rupture but collapsed and showed degenerative changes of the endothelium, probably due to lack of blood perfusion. The ventricular layer in exencephaly and spina bifida aperta was exposed to the amniotic cavity due to non closure of the neural tube. On day 17, this superficial lining of the primitive nervous tissue was disrupted by the expanding hemorrhages and subsequent necrosis. As a result vast amounts of fetal blood and cell debris were extruded into the amniotic fluid. During days 18 to 20, the degeneration of the nervous tissue proceeded rapidly. This process showed the same features in the ventricular cells, the primitive neurons and the neurons. Initially it was characterized by condensation of the nuclear chromatin and the cytoplasm, irregular outlines and breakdown of the plasma membrane. Only part of the cell debris was phagocytozed by macrophages.

It is concluded that the leakage of fetal serum and cell debris causes the elevation of fetal protein levels in the amniotic fluid of fetuses with open neural tube defects.

After the finding of elevated α-fetoprotein (AFP) levels in the human amniotic fluid of fetuses with neural tube defects, prenatal detection of these malformations became possible (Brock and Sutcliffe, '72). In the following years several reports were published on the clinical aspects of this diagnostic technique (Brock and Scrimgeour, '72; Brock et al., '75). These papers mainly described the reliability, sensitivity and prognostic value of this test in high risk pregnancies. However, in these clinical studies no fundamental work was done on the origin of the elevated AFP levels. Some investigators (Seppala and Ruoslahti, '74; Harris et al., '74) suggested that cerebrospinal fluid containing AFP leaked into the amniotic cavity from the open nervous system and from the exposed choroid plexus. In other papers increased fetal production (Seller et al., '74) and renal excretion of AFP were proposed (Gitlin and Boesman, '66). Brock and Sutcliffe ('72) proposed the possibility that in cases of neural tube defects AFP reaches the amniotic cavity from the associated skin defects. Because of these rather controversial hypotheses we decided to investigate this problem.

From studies of human anencephalic and spina bifida aperta fetuses it is well known that the open neural tube undergoes extensive

Received Feb 28, '78 Accepted Oct 18, '78

TABLE 1

Pregnancy outcome in untreated, hypervitaminosis A and trypan blue groups

Treatment	Pregnant dams	Living fetuses (mean)	Exencephalic (%)	Spina bifida (%)	Resorptions (% of implant)
None	18	182 (10 1)	0	0	14 (7 2)
Hypervitaminosis A	30	203 (6 7)	23 (11 3)	1 (0 5)	115 (36 0)
Trypan blue	24	160 (6 7)	0	25 (15 0)	110 (40 0)

degeneration resulting in the typical aspect seen at birth. Exencephaly, which is characterized by protrusion of cerebral tissue, can be considered as an early stage in the development of human anencephaly (Hanaway and Welch, '70). However, no detailed observations of the course of this process have been reported. To find out whether this progressive course of degeneration is related to the elevated AFP levels found in certain stages of gestation, an experimental study in rats was started. Exencephaly was induced by hypervitaminosis A (Giroud and Martinet, '56; Cohlan, '54), and spina bifida aperta was produced by trypan blue treatment (Gillman et al., '48).

In the present longitudinal investigation detailed light microscopical and ultrastructural studies were performed. Simultaneously amniotic fluid samples were investigated to find out whether a relation exists between the AFP level and the degree of degeneration. These results will be published separately (Peters and Hagenaars, '78).

MATERIALS AND METHODS

SPF-derived Wistar/RIV rats were housed in conventional environment in macrolon cages. Food pellets (Muracon, Trouw and Co., Putten, The Netherlands) and tap water were available ad libitum.

After a 14 days acclimatization period the rats (200-250 g) were mated one to one overnight. At 09.00 A.M. (= day 0) vaginal lavages were examined for sperm. The females were aselectively divided into three groups. Untreated animals (24) served as controls. For the induction of exencephaly 36 rats were given orally 40,000 I.U. vitamin A (Arovit®) daily on days 7-12. To induce spina bifida aperta 24 females were injected subcutaneously with 2 ml 1% aqueous trypan blue (BDH-stains 34078) daily on days 7-9.

On day 15 through day 20 laparotomy was performed under ether anaesthesia and the uterus was opened carefully at the antimesometrical side. The fetuses were removed and grossly examined externally and those showing gross malformations were selected for light microscopy (LM) and transmission electron microscopy (TEM).

Small blocks of tissue were excised from the head and the lumbar region and immersed in Karnovsky's fixative (Karnovsky, '65) for 6 hours. From day 17 on the fetuses were prefixed by cardiac perfusion with the fixative, diluted 1:1 with a phosphate buffer. After dehydration relatively large blocks were embedded in butylmethacrylate and cut at 1 μm. The sections were stained with toluidine blue and studied with a light microscope. For TEM the specimens were additionally postfixed with 1% OsO_4 for 3 hours, dehydrated, and embedded in Epon 812. Ultrathin sections were cut with a LKB ultramicrotome, double stained with uranyl acetate and lead citrate, and examined with a Philips EM 200 electron microscope.

RESULTS

Gross inspection

The mean number of implantations is the same in all groups but the number of living fetuses is drastically decreased in the treated groups (table 1). This reduction is associated with an increase in the number of resorptions.

In the control fetuses no neural tube malformations are detected. In the vitamin A group 23 cases of exencephaly and one case of spina bifida aperta are found. Furthermore 20 encephaloceles and 2 meningoceles are observed. In the trypan blue group 25 fetuses have spina bifida aperta, 10 have meningoceles and two have encephaloceles.

Spinal cord

The spinal cord of control fetuses has a wide zone of ventricular cells,[4] which are oriented perpendicularly to the lumen (fig. 1). This zone contains several round mitotic cells at

[4] The terminology used is according to the Boulder Committee ('70) Anat Rec Vol 166

the luminal surface (fig 2) The rest of the ventricular cells are elongated and their dark granular nuclei are situated at varying dis tances from the lumen At the luminal surface the cells are interconnected by means of junc-tions (fig 3) The cells are closely packed and there is little extracellular space In the ante-rior horn most of the neuroblasts are large and have distinct Nissl substance Their nuclei are pale and contain a conspicuous nucleolus (figs 4,5) In the posterior horn the neuroblasts are relatively small (figs 6, 7) At the periphery of the spinal cord the white matter is expanding The spindle shaped dark nuclei of oligo-dendrocytes can be observed between the de-veloping nerve fibers (fig 8) The anterior and posterior nerve roots have already been formed and the spinal ganglia are situated on either side of the primitive spinal cord Nu merous capillaries penetrate into the nervous tissue

Cerebral cortex

At day 15 the cerebral cortex of control fetuses consists of three distinct cell layers (fig 11) The ventricular zone consists of round mitotic cells bordering the lumen and of columnar cells oriented perpendicularly to the lumen with their nuclei at varying distances from the ventricular surface The intermedi-ate zone contains numerous polygonal and oval neuroblasts originating from the ven-tricular layer In addition narrow strands of cells with dark spindle shaped nuclei are pres-ent (fig 13) A narrow marginal zone is located at the periphery During the following days extensive proliferation and migration takes place so that on day 20 five different cell layers are present The ventricular layer has become very narrow The subventricular zone consists of polygonal neuroblasts and the in-termediate zone contains large round neuro-blasts with numerous cell processes (figs 12, 14) A thick cortical plate has developed This newly formed cell layer consists of oval shaped and elongated neuroblasts forming a palissade arrangement (fig 15) The marginal layer only has a few cell bodies but contains many cell processes

Neural tube defects

Vitamin A induced exencephaly is charac-terized by non closure of the cephalic part of the neural tube As a result the primitive brain is severely malformed (figs 9, 10) Nevertheless the histologic differentiation is quite normal, and several parts of the mal formed brain even show a striking resem blance to the structures in the control fetuses The infundibular part of the third ventricle and the cerebral cortex can clearly be iden tified

The induced spina bifida is characterized by absent closure of the neural tube in the lum bar region, consequently the nervous tissue still has more or less the shape of a primitive neural plate (fig 16) Despite this gross abnor-mality, the histologic differentiation in this stage is quite normal The matrix layer is pres-ent and numerous neurons as well as the pri-mordial white matter can be identified The anterior and posterior nerve roots and the spinal ganglia also have been formed

From day 15 on a progressive degeneration of the neural tissue takes place resulting in a complete hemorrhagic necrosis Although there are extensive differences in the anat-omy of exencephaly and spina bifida the de-generative processes show essentially the same features at the cellular and ultrastruc-tural level Therefore in the following para-graphs the events occurring in both regions will be described together

By day 15 perivascular edema begins to de velop (fig 17) and the matrix layer cells be-come separated by wide clefts During the following days this process continues, many capillaries are congested and the endothelial lining becomes extremely thin Then the vas-cular walls rupture and numerous blood cells penetrate into the surrounding tissue (figs 18, 19) At the same time the lumina of neigh-bouring capillaries become extremely narrow, so that they seem to be closed in light micros-copy However, the electron microscope dem-onstrates that the endothelium is swollen and almost closes the lumen with numerous tiny projections (fig 20) The plasma membrane is so irregular that small compartments seem to be cut off from the lumen (fig 21) The endo-thelial nuclei assume a scallopy shape and the cytoplasm contains many small vacuoles of medium density

The nucleus of the normal neuroblast is granular with small accumulations of chro-matin at the nuclear membrane Large num-bers of free ribosomes are present, arranged in rosettes or as polyribosomes Mitochondria, rough endoplasmic reticulum (RER) and some Golgi cisternae can be observed in the cyto-plasm (fig 22) In fetuses with induced neural tube defects the degeneration of the neuro-

blasts starts on day 17. First, the nuclear chromatin looses its granular structure, becomes condensed and accumulates at the nuclear membrane. The polyribosomal arrangement also disappears and the ribosomes become dispersed in the cytoplasm (fig. 23). The cellular outlines are irregular and the plasma membrane is locally disrupted. During the progressive degeneration, the mitochondria become swollen and the cisternae of the RER and the perinuclear cisternae dilate. An occasional myelin figure appears in the cytoplasm (fig. 24). In the further course of degeneration the nucleus and the cytoplasm becomes increasingly dense. The plasma membrane and the organelles break down rapidly and the debris is scattered throughout the intercellular space. In the final stage only a round dark nucleus remains, surrounded by a narrow rim of dense degenerated cytoplasm (fig. 25). Finally, fragmentation and complete lysis of the nucleus and cytoplasmic remnants takes place.

Thus it can be concluded that a distinct type of cell death takes place in large areas resulting in a complete destruction of the nervous tissue.

However, another type of cell death occurs, but to a much lesser degree. Throughout the ventricular and neuroblast cell populations, an occasional nucleus becomes round and its chromatin more sparse (fig. 26). The perinuclear cistern is enlarged. Simultaneously, the cytoplasm assumes a conspicuous watery appearance. The major part of the plasma membrane is intact but locally some small ruptures appear. The number of ribosomes is greatly reduced but they are still arranged in normal patterns. The RER cisternae are dilated and sometimes disrupted. The mitochondria begin to condense and loose their cristae. The Golgi system is also affected but only to a moderate degree. Many vesicles and loose membranes are scattered throughout the cytoplasm. Finally the cell membranes break down completely, the cytoplasm is fragmented and at last lysis of the naked nucleus takes place.

Thus it can be concluded that there are two types of cell degeneration: the predominant type is characterized by cytoplasmic and nuclear condensation, whereas the other type is mainly distinguished by loss of density.

Due to the rapidly increasing haemorrhages and necrosis present by day 17, the superficial ventricular zone is disturbed and disrupted (fig. 19). Initially a few blood cells and some debris are extruded into the amniotic cavity, but as the degeneration of the nervous tissue proceeds increasing amounts of fetal material contaminate the amniotic fluid.

As necrosis goes on during days 15-20, several macrophages appear. These large cells with large folded nuclei contain many vacuoles of varying size which contain remnants of erythrocytes, dense osmiophilic material and lipids (fig. 27).

DISCUSSION

Animal experiments are of great value to understand complicated developmental disorders. Whereas only a limited number of malformed human fetuses is available, large numbers of fetuses can be obtained in animal experiments. Thus it is feasible to perform extensive studies of consecutive developmental stages of malformations.

In the present study experiments with rats were performed to examine the mechanism that produces the elevation of α-fetoprotein levels in the amniotic fluid of fetuses with neural tube defects. In these experiments two well known teratogens, hypervitaminosis A and trypan blue, were used to induce exencephaly and spina bifida. In addition to these neural tube defects other malformations and resorptions can be induced by these two teratogenic agents (Gillman et al., '48; Cohlan, '54; Wilson, '55; Giroud and Martinet, '56).

The degenerative processes in the nervous tissue which take place from day 15 through day 20 begin with perivascular edema followed by rupture of blood vessels. This indicates that the degeneration of the fetal nervous tissue is caused by circulatory disturbances in the malformed brain and spinal cord. In the control fetuses the blood supply of the nervous tissue comes from an extensive adjacent capillary network which is fed by branches of the dorsal aortae. In the abnormal fetuses this network is also well developed but its supplying vessels form numerous tortuous sinusoids of varying diameter. This anomalous vascular system is embedded in loose mesenchyme with a low cell density. The vascular system apparently is not sufficient to sustain an adequate blood flow. The resulting congestion causes a poor transport of oxygen and nutrients to the developing tissues. This leads to edema, hemorrhage and degenerative changes and ultimately cell death.

Simultaneously with the disruption of capillaries other changes could be observed in neighbouring vessels with the electron micro-

scope. The endothelium of these capillaries showed an enormous number of slender projections into the lumen. There were also many small vacuoles in the endothelial lining. All these alterations produce narrowing or even complete obliteration of capillaries. The collapse of these capillaries probably results from a sudden decrease of perfusion caused by blood loss in the more proximal parts of the vascular system of the malformed nervous tissue.

The massive cell death which rapidly follows the vascular destruction shows the same features in matrix cells and in neurons. This type of cell degeneration is the same as that induced by some embryotoxic agents (Schweichel and Merker, '73), by hydroxyurea (Sadler and Cardell, '77), and by 5'-fluorodeoxyuridine (Langman and Cardell, '78).

One could suggest that the observed necrosis is caused by a direct deleterious influence of vitamin A or trypan blue. However, it has been demonstrated (Morriss, '73; Schweichel and Merker, '73) that hypervitaminosis A produces quite a different type of necrosis. Furthermore, it should be emphasized that in our study vitamin A and trypan blue treated fetuses showed the same cellular changes. Thus it can be assumed that the degeneration does not result from direct toxic effects of the administered agents on the nervous cells but that they are indirectly caused by circulatory insufficiency.

Contrary to the observations by Sadler and Cardell ('77) and Langman and Cardell ('78) no phagocytosis by surrounding ventricular cells took place. This can be explained by the fact that in massive necrotic areas none of the cells retains the capacity to phagocytose. Some phagocytosis did take place but this was done by macrophages probably originating from the blood.

In the past five years much work has been done on the prenatal diagnosis of anencephaly and spina bifida by means of AFP determinations. Fetal proteins have been demonstrated in the amniotic fluid of rat fetuses. Although amniotic AFP levels at day 20 are much higher than those at day 15, no differences were found in the amniotic fluid of control and exencephalic fetuses (Peters and Geelen, '75). However, on the intermediate days 16, 17 and 18, Smith and Kelleher ('77) found a significant difference of the AFP concentrations between exencephalic and control fetuses. Peters and Hagenaars ('78) measured amniotic AFP not only in exencephalic but also in spina bifida fetuses. This investigation also showed that amniotic AFP levels were significantly increased on days 16-19.

AFP is produced in early stages by the yolk sac endoderm and in later stages mainly by the fetal liver and gut. The AFP reaches the amniotic cavity by urinary excretion of normal fetuses (Weiss et al., '76).

In cases of neural tube defects the increase of amniotic AFP was thought to be caused by leakage of cerebrospinal fluid into the amniotic cavity (Brock, '76). However, Seller and Adinolfi ('75) demonstrated that the human cerebrospinal fluid and serum AFP in exencephalics were comparable to that of non malformed fetuses. This suggests that there is no elevated AFP synthesis in the abnormal fetuses. On day 15 the neural tube is open but there is no increase of amniotic AFP. However, immediately after the degeneration starts on days 16-17, a rise of AFP was measured in amniotic fluid (Smith and Kelleher, '77; Peters and Hagenaars, '78). This clearly demonstrates that leakage of cerebrospinal fluid is not the most important source of AFP elevation. It is more likely that the major contribution originates from the leakage of serum. Even the cell debris could contribute to this AFP elevation because it has been shown by Breborowicz and Mackiewicz ('77) that cultured human embryonic brain cells contain AFP. This hypothesis is also corroborated by the findings of Weiss et al. ('76) who stated that the amniotic AFP elevation was not due to the leakage of cerebrospinal fluid but by transsudation from any fetal defect.

As a final conclusion it can be stated that this experimental model demonstrates the morphologic basis of the amniotic AFP determination, which currently is in clinical use for the prenatal diagnosis of neural tube defects.

ACKNOWLEDGMENTS

The authors express their appreciation to Miss Francien Rotteveel, Mr. Theo Peters and Mr. Aart Verhoef for their excellent technical assistance, and Mr. Joop van Nesselrooij and Mr. Huub Kleintjes for the preparation of the photographs.

LITERATURE CITED

Breborowicz, J., and A. Mackiewicz 1977 The in vitro production of alphafoetoprotein by embryonic brain. Abstracts Vth Meeting Int. Res. Group for Carcinoembryonic Proteins. Copenhagen.

Brock, D. J. H. 1976 α-Fetoprotein and the prenatal diagnosis of central nervous system disorders. Child's Brain, *2:* 1-23.

Brock, D J H, and J B Scrimgeour 1972 Early prenatal diagnosis of anencephaly Lancet, *2* 1252 1253

Brock, D J H, J B Scrimgeour and M M Nelson 1975 Amniotic fluid alphafetoprotein measurements in the early prenatal diagnosis of central nervous system disorders Clin Genet, *7* 163 169

Brock, D J H, and R G Sutcliffe 1972 Alpha fetoprotein in the antenatal diagnosis of anencephaly and spinal bifida Lancet, *2* 197 199

Cohlan, S Q 1954 Congenital anomalies in the rat produced by excessive intake of vitamin A during pregnancy Pediatrics, *13* 556 557

Gillman, J, C Gilbert and T Gillman 1948 A preliminary report on hydrocephalus and spina bifida and other congenital anomalies in the rat produced by trypan blue S Afr J Med Sci *13* 47 90

Giroud, A, and M Martinet 1956 Teratogenese par hautes doses de vitamine A en fonction des stades du developpement Arch Anat Microsc Exp, *45* 77 98

Gitlin, D, and M Boesman 1966 Serum α fetoprotein, albumin and γG globulin in the human conceptus J Clin Invest, *45* 1826 1838

Hanaway, J, and G Welch 1970 Anencephaly a review and interpretation in terms of modern experimental embryology Dis Nervous Sys, *31* 527 533

Harris, R, R J Jennison, A I Barson, K M Laurence, E Ruoslahti and M Seppala 1974 Comparison of amniotic fluid and maternal serum alpha fetoprotein levels in the early antenatal diagnosis of spina bifida and anencephaly Lancet, *1* 429

Karnovsky M J 1965 A formaldehyde glutaraldehyde fixative of high osmolarity for use in electron microscopy J Cell Biol *27* 137a 138a

Langman, J, and E L Cardell 1978 Ultrastructural observations on FUdR induced cell death and subsequent elimination of cell debris Teratology *17* 229 270

Morriss, G M 1973 The ultrastructural effects of excess vitamin A on the primitive streak stage rat embryo J Embryol Morph, *30* 219 242

Peters, P W J, and J A G Geelen 1975 a_1 and a_2 fetoprotein levels in amniotic fluid of vitamin A induced anencephalic rat fetuses and in maternal serum Teratology, *12* 332

Peters, P W J, and A M Hagenaars 1978, in preparation

Sadler, T W, and R R Cardell 1977 Ultrastructural alterations in neuroepithelial cells of mouse embryos exposed to cytotoxic dosis of hydroxyurea Anat Rec, *188* 103 124

Schweichel, J U, and H J Merker 1973 The morphology of various types of cell death in prenatal tissues Teratology, *7* 253 266

Seller M J, and M Adinolfi 1975 Levels of albumin, alpha fetoprotein and IgG in human fetal cerebrospinal fluid Arch of Disease in Childhood, *50* 484-485

Seller, M J, J D Singer, T M Coltart and S Campbell 1974 Maternal serum fetoprotein levels and prenatal diagnosis of neural tube defects Lancet, *1* 428

Seppala, M, and E Ruoslahti 1974 Alpha fetoprotein and fetal development In L'Alphafoetoproteine R Masseyeff, ed INSERM, Paris, France, pp 387 391

Smith, C J, and P C Kelleher 1977 Alpha fetoprotein and albumin in experimentally induced exencephaly in the rat Teratology, *16* 71 78

Weiss, R R, J N Macri and K W Elligers 1976 Origin of amniotic fluid alpha fetoprotein in normal and defective pregnancies Obstet Gynecol, *47* 697 700

Wilson, J G 1955 Teratogenic activity of several azo dyes chemically related to trypan blue Anat Rec, *123* 313 333

PLATE 1

EXPLANATION OF FIGURES

1 Spinal cord of control fetus Day 15 × 32

2 Mitoses in ventricular zone on both sides of the central canal Control fetus, day 15 × 800

3 Ventricular cells connected by junctions (arrows) located at the lumen of the spinal cord Control fetus, day 15 × 7,600

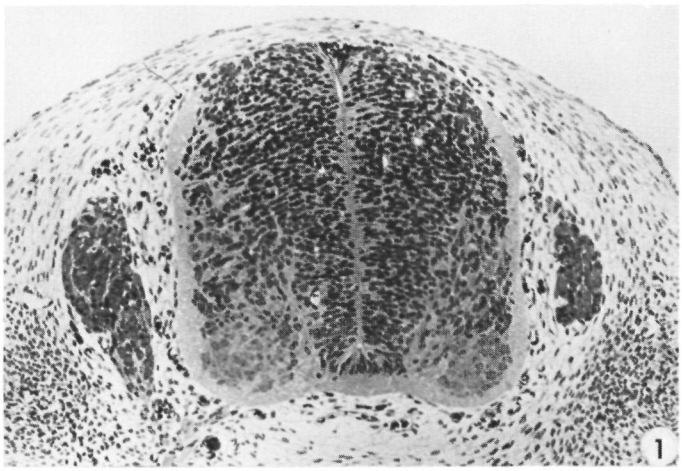
4 Anterior horn neurons and ventral nerve root (R) of the spinal cord * = white matter Control fetus, day 15 × 800

5 Large anterior horn neurons of the spinal cord with granular nuclei, numerous ribosomes, RER and small mitochondria Control fetus, day 15 × 4,600

6 Posterior horn neurons of the spinal cord * = white matter Control fetus, day 15 × 800

7 Small posterior horn neurons of the spinal cord with nuclei and a narrow rim of cytoplasm Control fetus, day 15 × 4,600

8 An oligodendrocyte with many vacuoles, ribosomes, RER and Golgi areas in the white matter of the spinal cord Control fetus, day 19 × 7,640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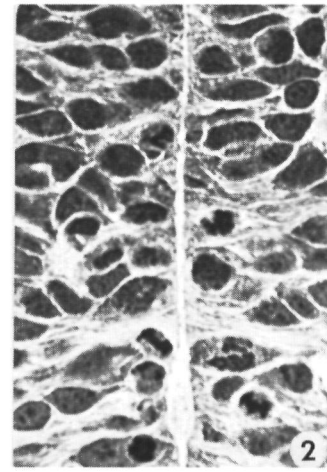
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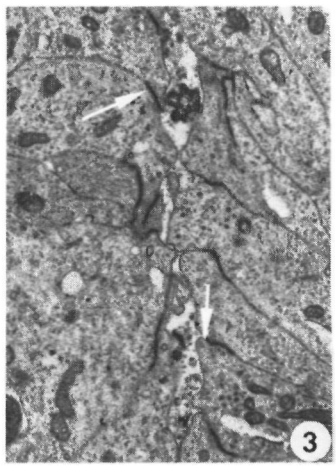
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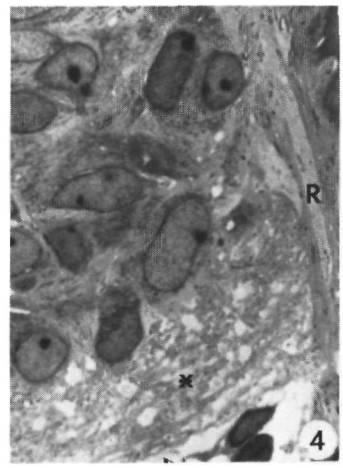
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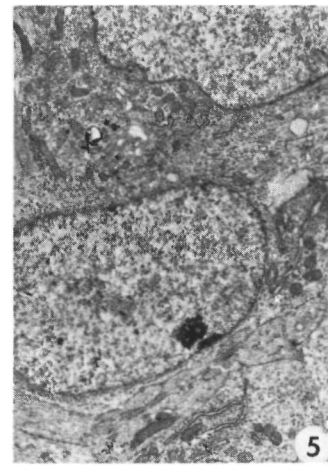
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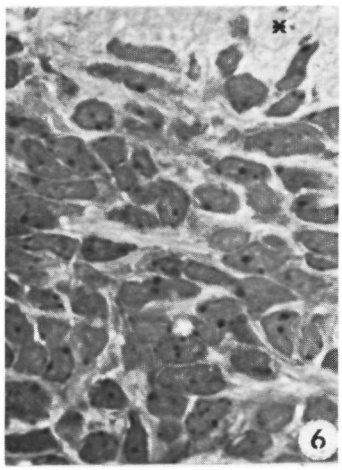
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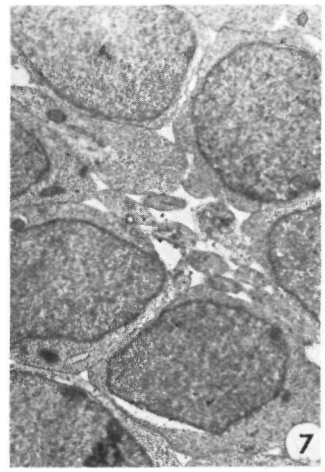
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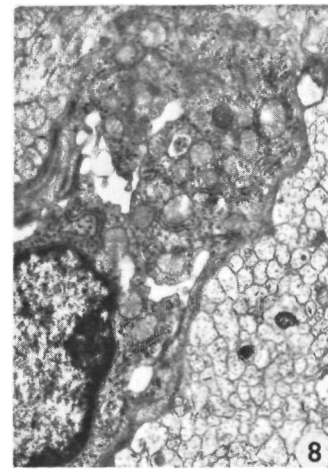
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PLATE 2

EXPLANATION OF FIGURES

9 Section of fetal head with lateral and third ventricles and plexus choroid Control fetus, day 15 × 8

10 Exencephalic fetus with everted neural tissue Vitamin A fetus, day 15 × 8

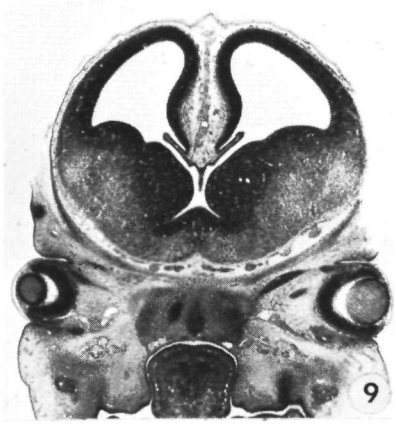
11 Cerebral cortex At the ventricular lumen (v) a broad ventricular zone (vz) is present The intermediate layer (i) and a narrow marginal zone (mz) are located further away from the ventricle Control fetus, day 15 × 200

12 Cerebral cortex on day 20 The layers are differentiated into a narrow ventricular zone (vz), subventricular zone (s), intermediate zone (i), cortical plate (c) and marginal zone (mz) Control fetus, day 20 × 80

13 Detail of figure 11 In the intermediate zone strands of very dark elongated cells are situated between polygonal and oval neuroblasts Control fetus, day 15 × 800

14 Detail of the intermediate zone with cell bodies, dendrites and axons Control fetus, day 20 × 800

15 Detail of the cortical plate with palissade arrangement of neurons Control fetus, day 18 × 800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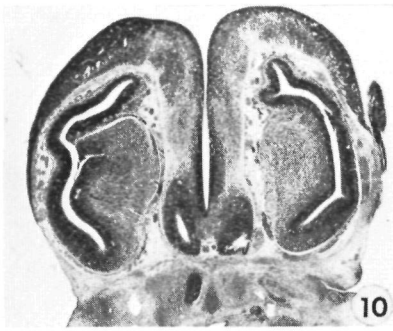
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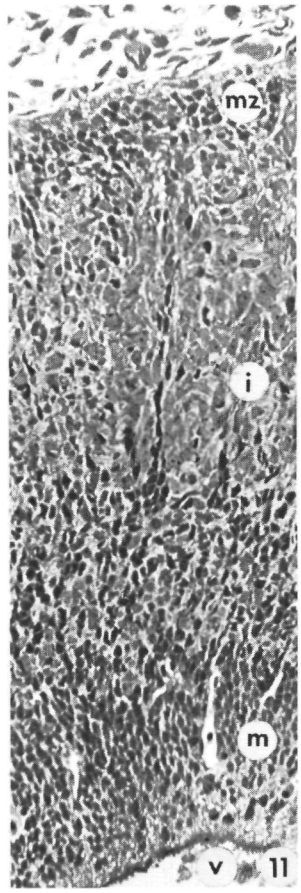
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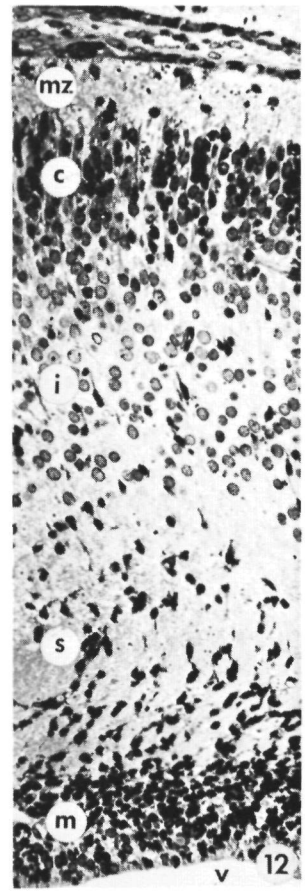
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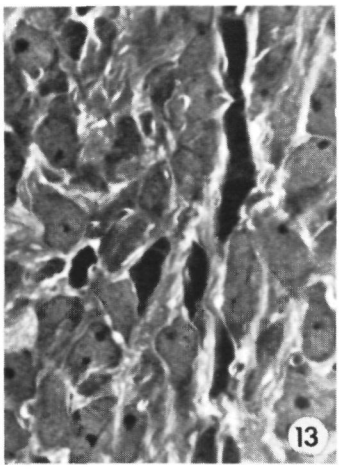
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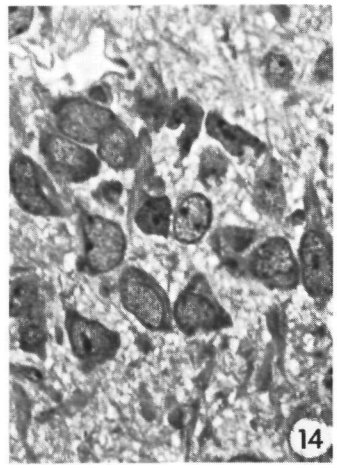
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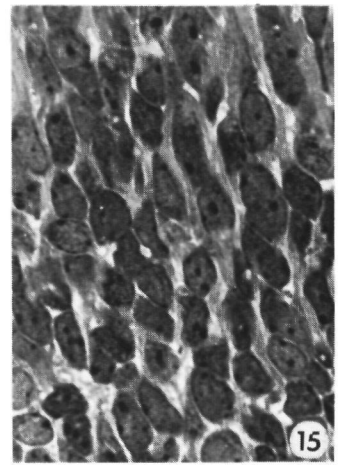
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PLATE 3

EXPLANATION OF FIGURES

16 Spina bifida aperta Trypan blue fetus, day 15 × 10

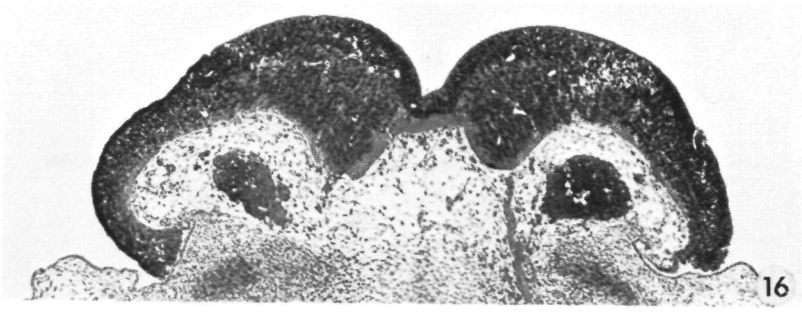
17 Edema in ventricular layer of spina bifida aperta Trypan blue fetus, day 15 × 800

18 Hemorrhages throughout the neural tissue of the spina bifida aperta Trypan blue fetus, day 15 × 320

19 Disruptions of the ventricular lining in spina bifida fetus Blood and cellular debris is leaking into the amniotic cavity Trypan blue fetus, day 17 × 800

20 Swollen endothelium of a blood vessel in the exencephalic tissue Vitamin A fetus, day 17 × 4,600

21 Blood vessel in the exencephalic tissue showing progressive degeneration of the endothelial cells Vitamin A fetus, day 19 × 7,600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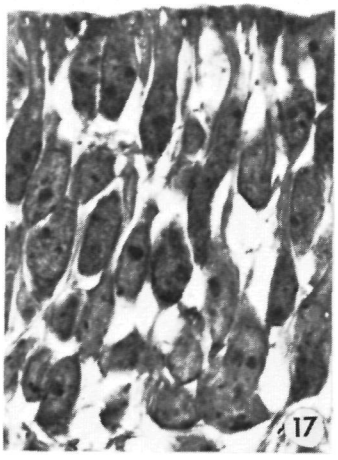
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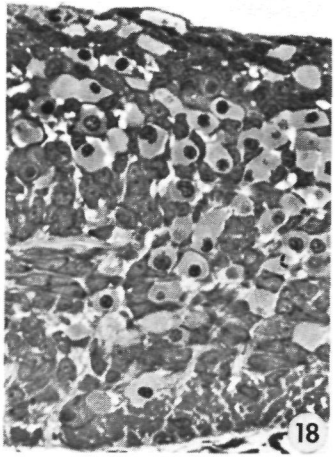
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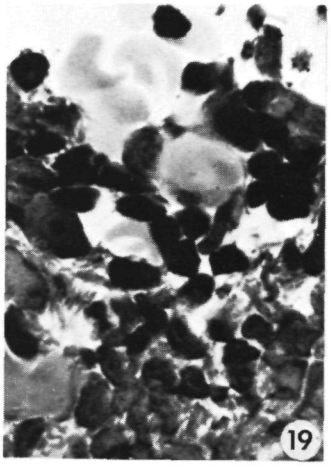
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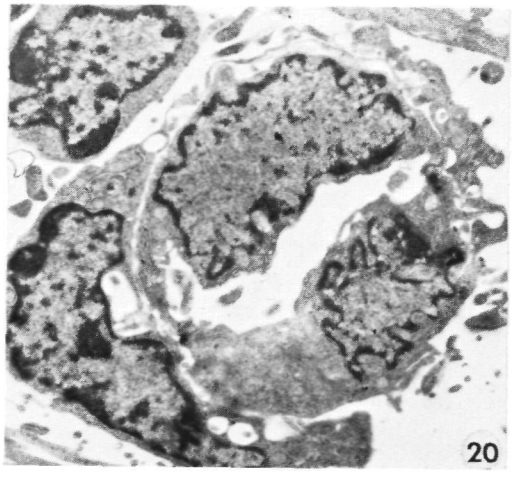
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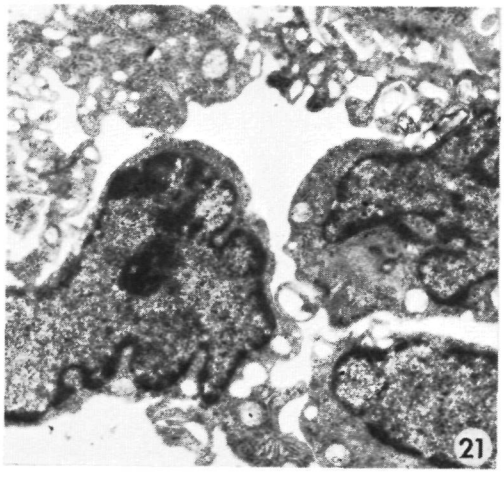
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PLATE 4

EXPLANATION OF FIGURES

22 A normal neuron with a granular nucleus and with some chromatin condensation at the nuclear membrane The cytoplasm is packed with ribosomes, mitochondria and RER Control fetus, day 16 × 14,700

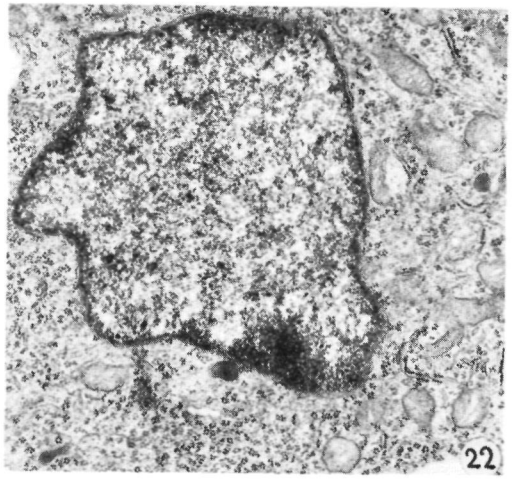
23 The initial stage of degeneration characterized by chromatin condensation The polyribosomal configuration disappears and the ribosomes are scattered throughout the cytoplasm Parts of the cell membrane are stripped off Vitamin A fetus, day 19 × 9,800

24 Further stage of degeneration showing a condensed chromatin at the periphery of a round nucleus Note swollen mitochondria, dilated cisternae of RER, myelin figures, lipid droplets, and disorganized ribosomes Vitamin A fetus, day 19 × 14,700

25 Different stages of neuronal degeneration showing varying degrees of nuclear condensation and cytoplasmic disruptions Vitamin A fetus, day 19 × 2,600

26 A second type of degeneration observed in only a few cells The nucleus becomes round and chromatin is sparse The perinuclear cystern is locally dilated In a watery cytoplasm condensed mitochondria, only a few ribosomes, some abnormal Golgi areas and many small vacuoles can be seen Vitamin A fetus, day 20 × 19,200

27 Remnants of phagocytized erythrocytes, dense osmiophilic material and lipids in large macrophages in degenerating spina bifida tissue Trypan blue fetus, day 20 × 3,100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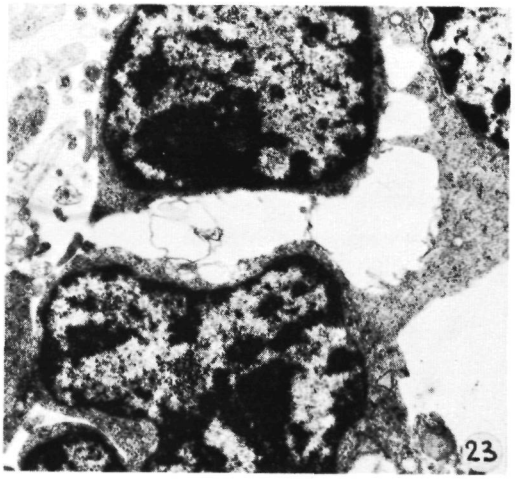
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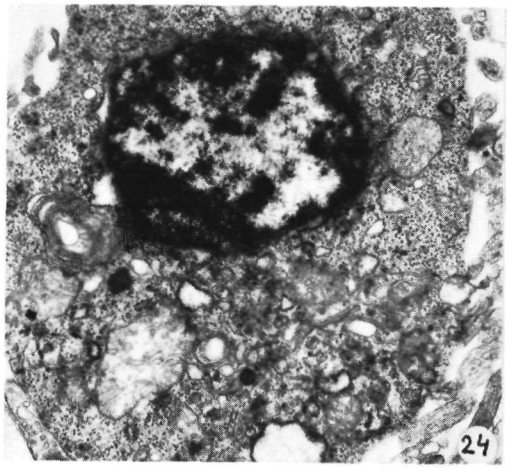
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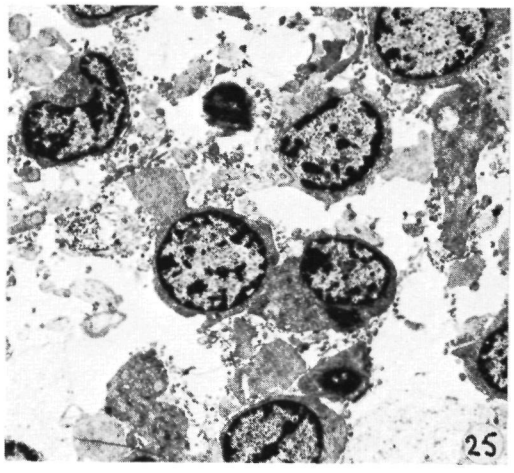
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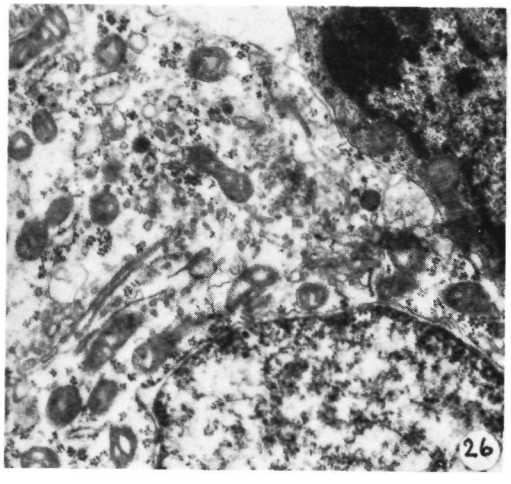
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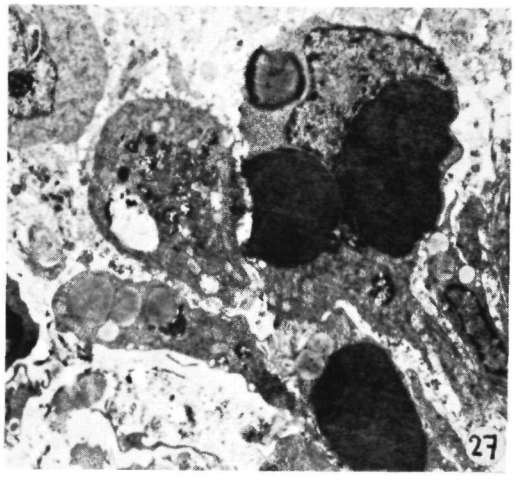
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SUMMARY

This thesis consists of six articles The first article is a review of the literature on congenital malformations induced by hypervitaminosis A This paper mainly focuses on the induction of defects of the closure of the neural tube In the second and third paper the normal closure of the cephalic part of the neural tube in the mouse embryo is examined at the light microscopical and ultrastructural level In the next two papers the effects of maternal administration of excess vitamin A on the closure of the neural tube are described The last article focuses on the later stages of development and describes the degeneration process in the nervous system after failure of neural tube closure The most important results of these investigations are summarized in the following paragraphs

I. Hypervitaminosis A induced teratogenesis (review of literature)

The first paper reviews the vitamin A induced malformations in the central nervous system and in various other organ systems, and discusses their morphogenesis and the susceptible developmental stages In addition the teratogenic effects in different species, the minimum effective dose, the interaction with various other substances, the influence of the way of administration and the pathophysiology are discussed The last paragraph reviews hypervitaminosis A and human pregnancy

II. Closure of the neural tube in the cephalic region of the mouse embryo

In mouse embryos varying in age from 9 to 20 somites the first closure of the neural groove was found to occur in the cervical region The fusion process gradually proceeded in rhombencephalic direction until it reached a level just caudal to the otic pits Shortly afterwards the prosencephalic walls fused together independent of the rhombencephalic closure This prosencephalic fusion process proceeded caudally in the direction of the mesencephalon until it reached the rostral portion of the rhombencephalon In addition the prosencephalic fusion proceeded in rostral direction toward the anterior neuropore, which was the last part of the brain vesicles to close Hence, the closure of the brain vesicles is not a continuous process proceeding from the rhombencephalon to the anterior neuropore, but occurs at several places at the same time

At the cellular level considerable differences in the fusion process were found between the various brain vesicles In the rhombencephalon the first bridge between the two opposing walls was formed by surface ectoderm and neural crest cells In the mesencephalon single squamous ectoderm and a few neuroepithelial cells established the first contact, whereas in the prosencephalon and the anterior neuropore region the apical ends of several neuroepithelial cells fused together to overbridge the gap between the opposing walls The surface ectoderm cells subsequently covered the neuroepithelial bridge

III. Ultrastructural observations on closure of the neural tube in the mouse

When the fusion of the neural walls in the cephalic part of mouse embryos varying in age from 9 to 20 somites was examined with the electron microscope, in the rhombencephalic region the rim of the neural wall was formed from outside inward by ectodermal surface cells, a row of flattened cells without surface projections and neuroepithelial cells At the junction of the surface ectoderm and the flat cells large cellular projections were seen The initial contact between the walls was made by the large cytoplasmic arms and numerous finger-like projections interdigitating with similar projections from the opposite wall The projections originated from the surface ectoderm and possibly neural crest cells During further fusion the surface ectoderm cells formed intercellular junctions

The initial contact in the mesencephalon was formed by extensions from the surface ectoderm and was followed by the formation of junctions The neuroepithelial cells facing the gap between the neural walls with their apical ends made contact with the cells from the opposing wall by numerous finger-like projections

The closing mechanism in the prosencephalon and anterior neuropore region differed from the other areas in that the initial contact was established by the neuroepithelial cells Only after this contact had been formed did the surface ectoderm cells close the gap In contrast with the other areas many phagocytosed particles were seen in the prosencephalon and in the region of the anterior neuropore Many particles from degenerated cells were found inside healthy surrounding cells Some of these particles contained nuclear material and cytoplasmic organelles

IV. Vitamin A-induced anomalies in young rat embryos

The normal development and the effects of hypervitaminosis A in 0 6 somite embryos (day 10 and 11 post coitum) were examined with the light microscope In the period that was studied the neural tube closed in the cervical region, the cephalic neural groove was formed and the cephalic mesenchyme developed However, it appeared that the density of the mesenchyme in the head region of the treated embryos decreased considerably in the 24 hour period after treatment Simultaneously big intercellular spaces developed It was concluded that the decrease of mesenchymal cells and the accompanying widening of the intercellular spaces resulted in insufficient support for the elevation of the cephalic neural walls and thus contributed to the failure of neural tube closure

V. The influence of excess vitamin A on neural tube closure in the mouse embryo

In this study mouse embryos were used, contrary to the preceding paper in which rat embryos were used Therefore one should remember that the development of the mouse embryo in the studied stages is about 24 hours ahead of that of the rat embryo The review of the literature shows that hypervitaminosis A causes failure of the closure of the neural tube in mice and rats

The effect of maternal administration of excess vitamin A on the closure of the rostral part of the neural tube was examined with light microscopy, transmission and scanning electronmicroscopy The embryos were treated with the vitamin on day 9 post coitum, just prior to the closure of the brain vesicles and examined during the following 24 hours, a period during which under normal conditions the brain completely closes In this period the number of somites increased from two to 20 At 18 24 hours after treatment the external features of the treated specimens differed from those of the controls The neural walls of the brain vesicles of the treated embryos had folded laterally and were widely separated, whereas the walls of the brain vesicles of the controls had folded dorsomedially and had fused in the midline Histologically, the first difference between treated and control embryos was noted at two hours after treatment, when large intercellular spaces were seen between the neuroepithelial cells of the treated embryos These spaces were mainly present between the apical ends of the wedgeshaped neuroepithelial cells In addition some of the neuroepithelial cells as well as some mesenchymal, endothelial, and surface ectoderm cells showed swelling and degeneration resulting from the vitamin A treatment This cell degeneration may contribute to failure of the neural tube to close due to loss of cohesion at the luminal surface and lack of mesenchymal support for the elevation of the neural walls The increase of intercellular spaces at the apical side of the neuroepithelium, however, was considered as the major cause of the failure of the neural tube to close

VI. Light microscopic and ultrastructural observations in advanced stages of induced exencephaly and spina bifida

In this investigation fetuses of days 15 20 with induced neural tube defects were used to study the further development of the malformed nervous system and to elucidate the morphologic basis of the elevation of fetal proteins in the amniotic fluid of fetuses with neural tube defects Pregnant rats were treated with hypervitaminosis A to induce exencephaly or with trypan blue to produce spina bifida aperta

On day 15 of gestation, edema developed in the primitive nervous tissue This was followed by the appearance of quickly expanding hemorrhages throughout the ventricular and intermediate zones Some capillaries did not rupture but collapsed and showed degenerative changes of the endothelium, probably due to lack of blood perfusion On day 17, the ventricular layer which was exposed to the amniotic cavity due to non closure of the neural tube was disrupted by the expanding hemorrhages and subsequent necrosis As a result vast amounts of fetal blood and cell debris were extruded into the amniotic fluid During days 18 to 20, the degeneration of the nervous tissue proceeded rapidly This process showed similar features in the ventricular cells, the primitive neurons and the neurons Initially it was characterized by condensation of the nuclear chromatin and the cytoplasm, irregular outlines and breakdown of the plasma membrane Only part of the cell debris was phagocytosed by macrophages

It was concluded that the leakage of fetal serum and cell debris causes the elevation of fetal protein levels in the amniotic fluid of fetuses with open neural tube defects

Reviewing the articles in this thesis we come to several conclusions Administration of excess vitamin A to pregnant experimental animals inhibited the closure of the rostral part of the embryonic neural tube This developmental disorder was investigated with morphological methods We did not extend our studies to the effects of excess vitamin A on the molecular level Examination of control and treated embryos revealed an increase of intercellular space in the cephalic mesenchyme Similar abnormalities were seen between the apical parts of the neuroepithelial cells Furthermore, some cellular degeneration occurred in the cephalic mesenchyme and neuroepithelium This vitamin A induced degeneration was clearly distinct from the physiological cell death which is present in controls as well as in treated embryos The walls of the neural groove of the treated specimens were pointing in dorsolateral direction, whereas in the controls they were bending in dorsomedial direction and eventually fused in the midline The failure of the closure of the neural tube was thought to be caused mainly by the increase of the intercellular spaces between the apical parts of the neuroepithelial cells The cell degeneration which occurred only sporadically in the neuroepithelium probably had only a minor effect The increased intercellular space and degeneration in the mesenchyme appeared to reduce support for the elevation of the neural walls and thus contributed to the maldevelopment of the brain Even though the embryonic nervous system had not closed it continued to differentiate However, after a few days the tissue rapidly degenerated and consequently the typical anencephalic features developed Contrary to the sporadic degeneration which occurred previously, this massive necrosis was thought to be caused by insufficient blood supply from the subjacent mesenchyme

Finally it can be concluded that morphologic methods can reveal minute initial disturbances that eventually lead to severe congenital malformations Unfortunately human malformed embryos are hardly available and seldom well preserved Therefore in future years the research on the development of congenital malformations shall have to rely heavily on experimental work The methods described in this thesis should then be used in combination with several other experimental approaches

SAMENVATTING

Dit proefschrift bestaat uit zes artikelen Het eerste artikel geeft een overzicht van de literatuur over aangeboren afwijkingen geinduceerd door hypervitaminose A Dit artikel richt zich met name op de inductie van stoornissen in de sluiting van de neurale buis In het tweede en derde artikel wordt de normale sluiting van het rostrale deel van de neurale buis in het muize-embryo beschreven met behulp van licht- en electronenmicroscopie In de daaropvolgende twee artikelen wordt het schadelijke effect van toediening van hoge doses vitamine A op de sluiting van de neurale buis beschreven De laatste publicatie richt zich op de latere ontwikkelingsstadia en beschrijft het degeneratieproces in het zenuwstelsel na het uitblijven van de sluiting van de neurale buis

De belangrijkste resultaten van deze onderzoekingen worden in de volgende paragrafen samengevat

I. De teratogene werking van hypervitaminose A (literatuuroverzicht)

Het eerste artikel in dit proefschrift presenteert een overzicht van de literatuur over door hypervitaminose A geinduceerde aangeboren afwijkingen in verschillende orgaansystemen, met name in het centraal zenuwstelsel, en bespreekt vervolgens de morphogenese en gevoelige ontwikkelingsstadia Bovendien worden besproken de teratogene effecten in verschillende diersoorten, de minimaal effectieve dosis, de interactie met verscheidene andere stoffen, de invloed van de toedieningswijze en de pathophysiologie De laatste paragraaf vermeldt de gevolgen van hypervitaminose A voor de zwangerschap bij de mens

II. De sluiting van de neurale buis in het kopgebied van het muize-embryo

In muize-embryonen, in ontwikkeling variërend van 9 tot 20 somieten, werd geconstateerd dat de sluiting van de neurale groeve het eerst optrad in het cervicale gebied Het fusieproces van de beiderzijde gelegen neurale wallen ging vervolgens geleidelijk voort in het rhombencephalon tot vlakbij de oorinstulpingen Spoedig daarna trad er fusie op van de neurale wallen van het procescephalon Dit proces vond plaats onafhankelijk van de fusie van de wallen van het rhombencephalon Het fusieproces in het prosencephalon ging in caudale richting voort naar het mesencephalon en verder naar het rostrale deel van het rhombencephalon Bovendien ging het in het prosencephalon verder in rostrale richting naar de regio van de neuroporus anterior Dit onderdeel van de hersenblaasjes sloot als laatste Dus de sluiting van de hersenblaasjes is niet een continu proces verlopend van rhombencephalon naar neuroporus anterior, maar vindt tegelijkertijd plaats op verschillende plaatsen

Op cellulair niveau bestonden er aanzienlijke verschillen in het fusieproces van de verschillende hersenblaasjes In het rhombencephalon werd de eerste verbinding tussen de tegenover elkaar gelegen wallen gevormd door oppervlakte-ectoderm en cellen van de neurale lijst In het mesencephalon vormden een enkele plaveiselcel van het ectoderm en enige neuro-epitheelcellen het eerste contact Daarentegen fuseerden in het prosencephalon en het neuroporus anterior gebied de apicale uiteinden van verscheidene neuro-epitheelcellen om de spleet tussen de beide neurale wallen te overbruggen Daarna werd deze neuro-epitheliale verbinding bedekt met oppervlakte-ectoderm

III. Ultrastructureel onderzoek van de sluiting van de neurale buis in de muis

Bij het electronenmicroscopisch onderzoek van de fusie van de neurale wallen in het kopgebied van muize-embryonen variërend in ontwikkeling van 9 tot 20 somieten bleek dat in het rhombencephalon de top van de neurale wal werd gevormd door achtereenvolgens oppervlakte-ectodermcellen, enige afgeplatte cellen zonder uitstulpingen van de celmembraan en tenslotte door neuro-epitheelcellen Op de overgang tussen oppervlakte-ectoderm en de afgeplatte cellen bevonden zich grote celuitlopers Het eerste contact tussen de tegenover elkaar liggende neurale wallen werd van weerszijden gemaakt door brede cytoplasma uitlopers en talrijke slanke korte uitlopers De uitlopers waren afkomstig van het oppervlakte-ectoderm en wellicht ook van cellen van de neurale lijst Gedurende het verdere verloop van het fusieproces vormden de cellen van het oppervlakte-ectoderm intercellulaire verbindingen

Het eerste contact in het mesencephalon werd gevormd door uitlopers van het oppervlakte-ectoderm, daarna ontwikkelden zich ook hier intercellulaire verbindingen De neuro-epitheelcellen maakten contact door middel van slanke uitlopers Het sluitingsmechanisme in het prosencephalon en in het gebied van de neuroporus anterior verschilde van dat in de bovenvermelde gebieden door het feit dat hier het eerste contact werd gelegd tussen neuro-epitheelcellen Pas nadat dit contact was gemaakt ontstond er een verbinding tussen de cellen van het oppervlakte-ectoderm van beide zijden In tegenstelling tot de andere gebieden werden in het prosencephalon en in de regio van de neuroporus anterior vele gephagocyteerde overblijfselen van gedegenereerde cellen aangetroffen in intacte naburige cellen Een aantal van deze gephagocyteerde partikels bestond uit delen van celkernen of organellen

IV. Door vitamine A geïnduceerde afwijkingen in jonge ratte-embryonen

De normale ontwikkeling en de effecten van hypervitaminose A op embryonen van 0 6 somieten (dag 10 en 11 post coitum) werden bestudeerd met lichtmicroscopie In de periode die werd bestudeerd vond sluiting plaats van de neurale buis in het cervicale gebied, de neurale groeve in het kopgebied begon zich te ontwikkelen en het kopmesenchym werd gevormd Het bleek echter dat bij embryonen van behandelde drachtige ratten de dichtheid van het mesenchym in het kopgebied aanzienlijk verminderde in de periode van 24 uur na de behandeling Tegelijkertijd ontwikkelden zich grote intercellulaire ruimtes Er werd geconcludeerd dat de afname van het aantal mesenchymcellen en de daarbij optredende verwijding van de intercellulaire ruimtes als gevolg had, dat in onvoldoende mate steun verleend werd aan het oprichten van de neurale wallen en bijdroeg tot het uitblijven van de sluiting van de neurale buis

V. Het effect van overdosering van vitamine A op de sluiting van de neurale buis in het muize-embryo

In deze studie werden muize-embryonen gebruikt en geen ratte-embryonen zoals in het voorgaande onderzoek Men dient daarom te bedenken dat de ontwikkeling van de muis in de door ons onderzochte stadia 24 uur vóór ligt op die van de rat Uit het literatuur overzicht blijkt dat hypervitaminose A zowel bij muis als rat stoornissen in de sluiting van de neurale buis veroorzaakt

Het effect van het toedienen van een overdosis vitamine A op het sluiten van het rostrale deel van de neurale buis werd bestudeerd met lichtmicroscopie, transmissie en scanning electronenmicroscopie De embryonen werden behandeld met vitamine A op dag 9 post coitum vlak voor de sluiting van de hersenblaasjes en onderzocht in de daaropvolgende 24 uur, een periode waarin normalerwijs de hersenaanleg volledig sluit In deze periode nam het aantal somieten toe van 2 tot 20 18 24 uur na de behandeling verschilden de uitwendige kenmerken van de embryonen duidelijk van die van de controlegroep De neurale wallen van de hersenaanleg van de behandelde embryonen waren naar lateraal gericht en ver van elkaar gescheiden, terwijl in de controle embryonen de neurale wallen daarentegen naar dorsomediaal waren gericht en fusie hadden

ondergaan in de mediaanlijn Het eerste histologische verschil tussen de behandelde en de controle embryonen werd reeds twee uur na de behandeling gezien en bestond uit het optreden van grote intercellulaire ruimtes tussen de neuro-epitheelcellen van de behandelde embryos Deze ruimtes kwamen voornamelijk voor tussen de apicale uiteinden van de wigvormige neuro-epitheelcellen Bovendien vertoonden enige cellen van het neuro-epitheel, mesenchym, endotheel en oppervlakte-ectoderm zwelling en degeneratie Dit celverval droeg mogelijkerwijs bij tot het uitblijven van de sluiting van de neurale buis ten gevolge van verlies van samenhang van de apicale uiteinden van de neuro-epitheelcellen en onvoldoende mesenchymale ondersteuning voor het oprichten van de neurale wallen De toename van de intercellulaire ruimtes in the apicale deel van het neuro-epitheel werd echter beschouwd als de belangrijkste oorzaak van het uitblijven van de sluiting van de neurale buis

VI. Lichtmicroscopische en ultrastructurele waarnemingen in gevorderde stadia van geïnduceerde exencephalie en spina bifida

In dit onderzoek werd de verdere ontwikkeling van het zenuwstelsel bestudeerd in foeten van dag 15 20 met geinduceerde sluitingsdefecten van de neurale buis Bovendien werd gezocht naar een morphologische basis voor de stijging van foetale proteines in het amnionvocht van foeten met anencephalie en spina bifida aperta In deze experimenten werden drachtige ratten behandeld met hypervitaminose A om exencephalie te induceren en met trypaanblauw om spina bifida aperta op te wekken

Op dag 15 ontstond er oedeem in het zich ontwikkelende zenuwweefsel Daarna verschenen er bloedingen in de ventriculaire en de intermediaire cellagen Een aantal capillairen ruptureerde niet maar collabeerde en vertoonde degeneratieve veranderingen van het endotheel, mogelijkerwijs ten gevolge van het ophouden van de bloed-doorstroming Op dag 17 werd de ventriculaire laag doorbroken door zich uitbreidende bloedingen en necrotische gebieden Aangezien deze ventriculaire laag in de foeten met sluitingsdefecten van de neurale buis aan de oppervlakte van de foeten ligt kwamen er grote hoeveelheden foetaal bloed en celdebris in de amnionholte Gedurende dag 18 20 schreed de degeneratie van het zenuwweefsel snel voort Dit proces toonde dezelfde morphologische kenmerken zowel in cellen van de ventriculaire laag als in neuronen van verschillende rijpingsstadia in de overige cellagen De degeneratie werd gekenmerkt door toename van de dichtheid van chromatine en cytoplasma, door onregelmatige cel-contouren en door het uiteenvallen van de celmembraan

Er werd geconcludeerd dat de stijging van de foetale proteines in het amnionvocht bij foeten met open neurale buis defecten wordt veroorzaakt door foetaal serum en celdebris afkomstig van het degenererend foetale weefsel

Wanneer we de verschillende artikelen in dit proefschrift overzien, zijn er een aantal conclusies te trekken Het was mogelijk door toediening van hoge doses vitamine A de sluiting van het rostrale deel van de neurale buis te verhinderen Bij het onderzoek van deze stoornis maakten we alleen gebruik van morphologische waarnemingen We zijn in het onderzoek niet ingegaan op de effecten van hoge doses vitamine A op moleculair niveau Bij onderzoek van controle en behandelde embryonen bleek spoedig na de behandeling een toename op te treden van de intercellulaire ruimte in het kopmesenchym In het neuro-epitheel was er eveneens een toename van intercellulaire ruimte, dit verschijnsel deed zich vooral voor tussen de apicale delen van de neuro-epitheelcellen

Bovendien werd in het neuro-epitheel en in het mesenchym enige door vitamine A geïnduceerde celdegeneratie aangetroffen, die duidelijk verschilde van de physiologische celdegeneratie die zowel in de controle embryonen als in de behandelde embryonen voorkwam De wallen van de neurale groeve in de behandelde dieren bleven naar dorso-lateraal gericht, terwijl ze in de controle dieren naar dorso-mediaal ombogen om tenslotte te fuseren in de mediaanlijn Het uitblijven van de vorming van de neurale buis werd voornamelijk verklaard door de toename van intercellulaire ruimtes tussen de apicale delen van de neuro-epitheelcellen De celdegeneratie die slechts plaatselijk optrad in het neuro-epitheel had waarschijnlijk slechts een geringe invloed De toename van de intercellulaire ruimte en de celdegeneratie in het mesenchym leken een verminderde steun te bieden voor het oprichten van de neurale wallen en droegen op deze wijze bij tot de sluitingsstoornis Het niet gesloten embryonale zenuwstelsel vertoonde een toenemende differentiatie Na enige dagen echter trad er uitgebreide degeneratie op met als resultaat dat zich het karakteristieke aspect van de anencephalie ontwikkelde In tegenstelling tot de enige dagen eerder opgetreden celdegeneratie werd deze massale necrose toegeschreven aan insufficiente bloedtoevoer vanuit het onderliggende mesenchym

Tot slot kan geconcludeerd worden dat het mogelijk is met verfijnde morphologische technieken in vroege embryonale stadia die afwijkingen op te sporen die de eerste aanzet vormen voor het ontstaan van congenitale misvormingen Humane embryonen met ontwikkelingsstoornissen zijn nauwelijks beschikbaar en zelden geschikt voor morphologische onderzoeksmethoden Daarom zal in de toekomst het onderzoek naar het ontstaan van aangeboren afwijkingen zich sterk moeten richten op experimenteel werk, waarbij de technieken, zoals beschreven in dit proefschrift, een onderdeel vormen van een uitgebreid arsenaal van onderzoeksmethoden

CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1947 te Roermond geboren Na het eindexamen Gymnasium b ging hij in 1965 geneeskunde studeren aan de Katholieke Universiteit te Nijmegen. Tijdens de doctoraal studie was hij part-time werkzaam op het Laboratorium voor Psychopathologie en Neurophysiologie (hoofd. H H. J Jaspar, zenuwarts) van het Instituut voor Neurologie (hoofd wijlen Prof. dr J J G. Prick) voor het verrichten van experimenteel onderzoek naar het ontstaan van aangeboren afwijkingen Na het behalen van het doctoraal examen in 1971 zette hij zijn werkzaamheden op genoemd laboratorium voort. In 1974 werd het artsexamen behaald. Hierna was hij tot 1976 verbonden aan het Elisabeth Ziekenhuis en het Maria Ziekenhuis te Tilburg (neurologen. J. H van Luijk, Prof dr B P. M. Schulte, opleider, A C M Leyten en dr C. W. G. M Frenken) in het kader van de opleiding tot zenuwarts. In deze periode werd bovengenoemd onderzoek voortgezet, nu mede in samenwerking met de Afdeling Teratologie (hoofd drs. P W. J. Peters) van het Rijksinstituut voor de Volksgezondheid te Bilthoven. Van juli 1976 tot juli 1977 werkte hij bij Prof. J. Langman M D , Ph D. op het Department of Anatomy, Medical School, University of Virginia, Charlottesville, U S A , met financiele steun van een IBRO Fellowship Neurobiology. Vervolgens werd de specialisatie voortgezet aan het Instituut voor Neurologie, St. Radboud Ziekenhuis te Nijmegen (opleider: wijlen Prof. dr. J. J G. Prick) Hij werd in 1977 medewerker van de Werkgroep Quantitatief Hersenschorsonderzoek (voorzitter· Prof dr F. J. M. Gabreels) Sinds 1978 is hij in het kader van zijn specialisatie werkzaam op het Instituut voor Psychiatrie, St Radboud Ziekenhuis te Nijmegen (opleiders Prof dr. S. J Nijdam en Prof. dr. A. A Fischer)

STELLINGEN

behorend bij het proefschrift

THE TERATOGENIC EFFECTS OF HYPERVITAMINOSIS A
ON THE FORMATION OF THE NEURAL TUBE

J A G GEELEN

I

Onderzoek van geïnduceerde aangeboren misvormingen bij proefdieren is noodzakelijk voor het verkrijgen van inzicht in de ontstaanswijze van congenitale afwijkingen bij de mens.

II

Teratologisch onderzoek kan een belangrijke bijdrage leveren tot het begrijpen van de normale embryonale ontwikkelingsprocessen

III

Aangezien onderzoek en behandeling van aangeboren afwijkingen tegenwoordig wordt verricht door verschillende specialismen, moet getracht worden de betrokken artsen en wetenschapsbeoefenaars te verenigen om de vele teratologische problemen op te lossen

J Warkany, Teratology 1979(20)204

IV

Voor het beoordelen van de teratogeniciteit van geneesmiddelen zijn behalve *in vivo* ook *in vitro* experimenten gewenst

V

De term phakomatose als verzamelnaam voor tubereuse sclerose, neurofibromatose, de ziekte van Hippel-Lindau en de ziekte van Sturge-Weber suggereert ten onrechte dat deze aandoeningen wezenlijke gemeenschappelijke kenmerken hebben

VI

Hersenbiopsieën ter diagnose van degeneratieve hersenziekten dienen alleen verricht te worden in centra die over uitgebreide morphologische en biochemische faciliteiten beschikken

VII

Een stage in de neuroanatomie en de neuropathologie mag in de opleiding tot neuroloog niet ontbreken

VIII

Bij de spoorbrug in de Nijmeegse benedenstad behoort een beeld te worden geplaatst van Japi, de Uitvreter uit het werk van Nescio

Nijmegen, 23 juni 1980